Analogues of the Potent Nonpolyglutamatable Antifolate N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523) with Modifications in the Side Chain, *p*-Aminobenzoyl Moiety, or 9,10-Bridge: Synthesis and in Vitro Antitumor Activity

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Seven N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (**2**, PT523) analogues were synthesized by modifications of the literature synthesis of the corresponding AMT (**1**) analogues and were tested as inhibitors of tumor cell growth. In growth assays against cultured CCRF-CEM human leukemic cells exposed to drug for 72 h, the IC_{50} values of analogues in which N^{10} was replaced by CH_2 and CHMe were found to be 0.55 ± 0.07 and 0.63 ± 0.08 nM, and thus these analogues are more potent than **1** (IC_{50} = 4.4 ± 1.0 nM) or **2** (IC_{50} = 1.5 ± 0.39 nM). The 10-ethyl-10-deaza analogue of **2** (IC_{50} = 1.2 ± 0.25 nM) was not statistically different from **2** but was more potent than edatrexate, the 10-ethyl-10-deaza analogue of **1**, which had an IC_{50} of 3.3 ± 0.36 nM. In contrast, the analogue of **2** with both an ethyl and a CO_2Me group at the 10-position had an IC_{50} of 54 ± 4.9 nM, showing this modification to be unfavorable. The 4-amino-1-naphthoic acid analogue of **2** had an IC_{50} of 1.2 ± 0.22 nM, indicating that replacement of the *p*-aminobenzoic acid (pABA) moiety does not diminish cytotoxicity. The analogues in which the $(CH_2)_3$ side chain was replaced by slightly longer CH_2SCH_2 and $(CH_2)_2$-SCH_2 groups gave IC_{50} values of 4.4 ± 1.1 and 5.0 ± 0.56 nM and thus were somewhat less potent than the parent molecule. However the analogues in which the aromatic COOH group was at the meta and para positions of the phthaloyl ring had IC_{50} values of 7.5 ± 0.47 and 55 ± 0.07 nM, confirming the low potency we had previously observed with these compounds against other cell lines. Overall, the results in this study support the conclusion that, while the position of the phthaloyl COOH group and the length of the amino acid side chain in **2** are important determinants of cytotoxic potency, changes in the pABA region and 9,10-bridge are well-tolerated and can even increase potency.

Introduction

Analogues of the classical dihydrofolate reductase (DHFR) inhibitor aminopterin (AMT, **1**; cf. Chart 1) in which N^{δ}-hemiphthaloyl-L-ornithine replaces the glutamic acid side chain exhibit potent in vitro antitumor activity despite the fact that, unlike the glutamate derivatives, they are incapable of being metabolized to more tightly enzyme-bound and less rapidly effluxed γ-polyglutamates (reviewed in refs 1 and 2). Detailed investigations of the mode of action of N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523, **2**; cf. Chart 1), the first compound synthesized in this series,[3,4] established that the drug is a potent inhibitor of both purine and pyrimidine biosynthesis,[5] that it is taken up, at least in part, via the reduced folate carrier (RFC),[5] and that its activity is associated with rapid and persistent depletion of cellular tetrahydrofolate cofactors, both in cultured cells[5] and in mice.[6] It is a moderately good substrate for partially purified human hepatic aldehyde oxidase,[7] which converts it to a 7-hydroxy derivative. Although some 7-hydroxylation has indeed been observed in tumor-bearing mice,[6] the amide bond in the hemiphthaloyl group appears to be stable in plasma and tissues. In the presence of acid, on the other hand, **2** is extensively cleaved to the weak DHFR inhibitor N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine.[8,9] The high potency of **2** against cultured cells is believed to be due to a combination of two factors that work together in its favor: (a) an unusually strong interaction with DHFR, a process in which the phthaloyl group is believed to play a specific and important structural role,[10,11] and (b) an unusually efficient utilization of the reduced folate carrier (RFC) for influx into cells.[12−14]

Because of the novel mode of action of **2** as a water-soluble nonpolyglutamatable DHFR inhibitor, a systematic program of synthesis of second-generation analogues was undertaken in our laboratory with a view to identifying structure−activity correlations among this class of antifolates. Previous papers have described analogues **3**−**14**, in which (a) the side chain was either lengthened or shortened (**3**−**5**),[15,16] (b) the carboxyl group in the phthaloyl moiety was moved from the ortho to the meta or para position (**6**, **7**),[16] (c) ring substitution was introduced into the 4-aminobenzoic acid (pABA) moiety (**8**),[16] and (d) the B-ring was modified at N^5 and/or N^8 (**9**−**13**).[17] In the present paper we report the synthesis of additional analogues (**15**−**21**) in which N^{10} is replaced by carbon (**15**−**18**), the pABA moiety is replaced by 4-amino-1-naphthoic acid (**19**), or the side

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Chart 1

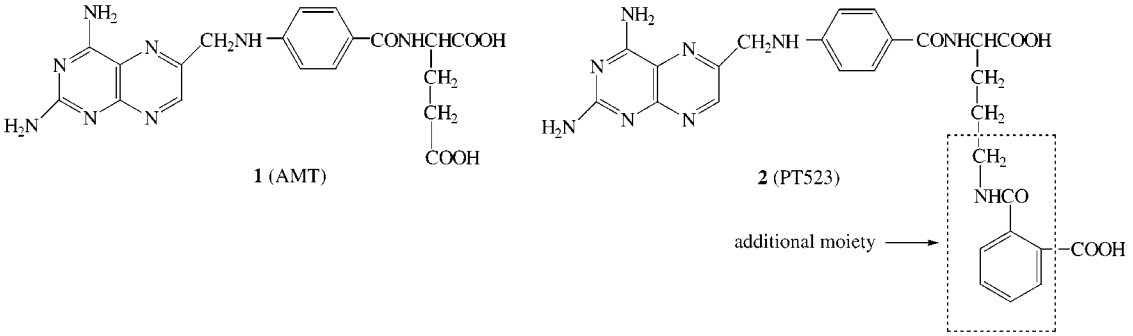
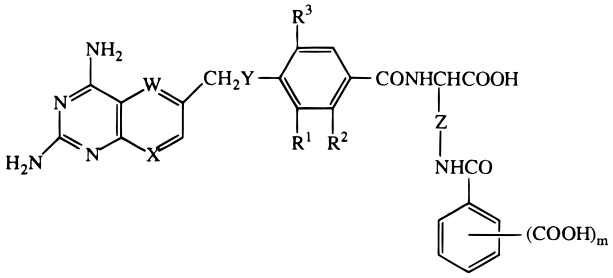


Chart 2



Cmpd	W	X	Y	R^1	R^2	R^3	Z	m
3-5	N	N	NH	H	H	H	$(CH_2)_{1,2,4}$	ortho
6, 7	N	N	NH	H	H	H	$(CH_2)_3$	meta, para
8	N	N	NH	Cl	H	Cl	$(CH_2)_3$	ortho
9,10	CH(Me)	N	NH	H	H	H	$(CH_2)_3$	ortho
11	N	CH	NH	H	H	H	$(CH_2)_3$	ortho
12-14	CH(Me,Cl)	CH	NH	H	H	H	$(CH_2)_3$	ortho
15	N	N	CH_2	H	H	H	$(CH_2)_3$	ortho
16,17	N	N	CH(Me,Et)	H	H	H	$(CH_2)_3$	ortho
18	N	N	$C(Et)CO_2Me$	H	H	H	$(CH_2)_3$	ortho
19	N	N	NH	$(CH=CH)_2$		H	$(CH_2)_3$	ortho
20	N	N	NH	H	H	H	CH_2SCH_2	ortho
21	N	N	NH	H	H	H	$(CH_2)_2SCH_2$	ortho

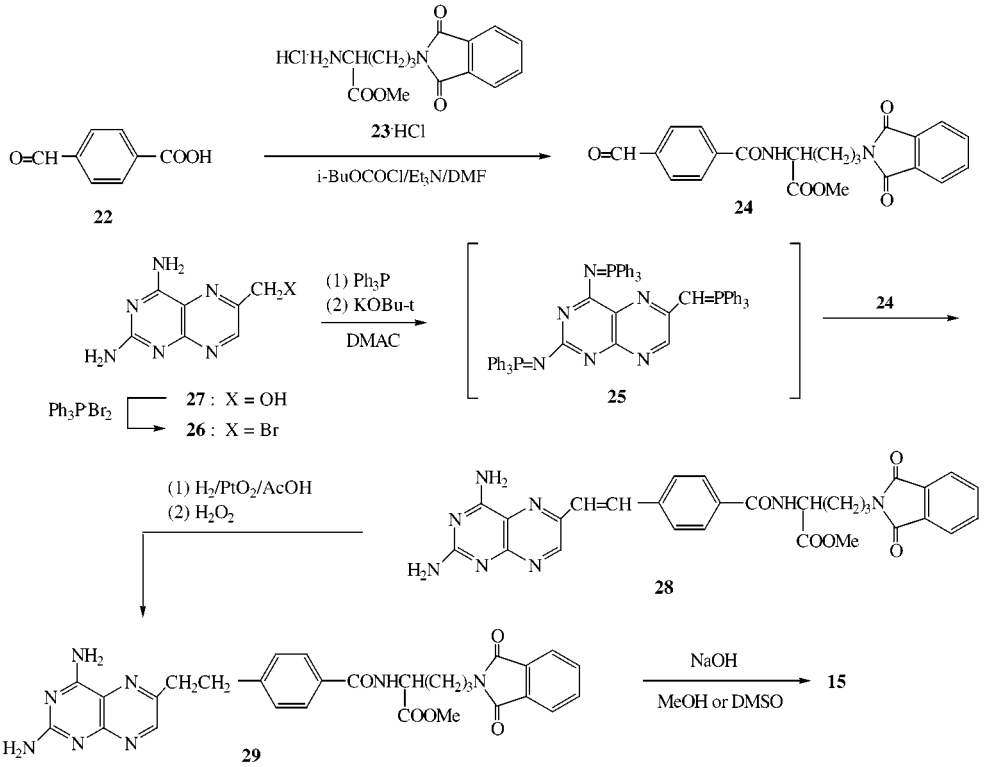
chain contains a sulfur atom in the form of cysteine or homocysteine (**20**, **21**). Compound **17** may be viewed as an analogue of 10-ethyl-10-deazaaminopterin (edatrexate),[18] which has been undergoing clinical trial for the past several years.[19] The structures of the compounds **3**−**21** are shown in Chart 2.

Chemistry

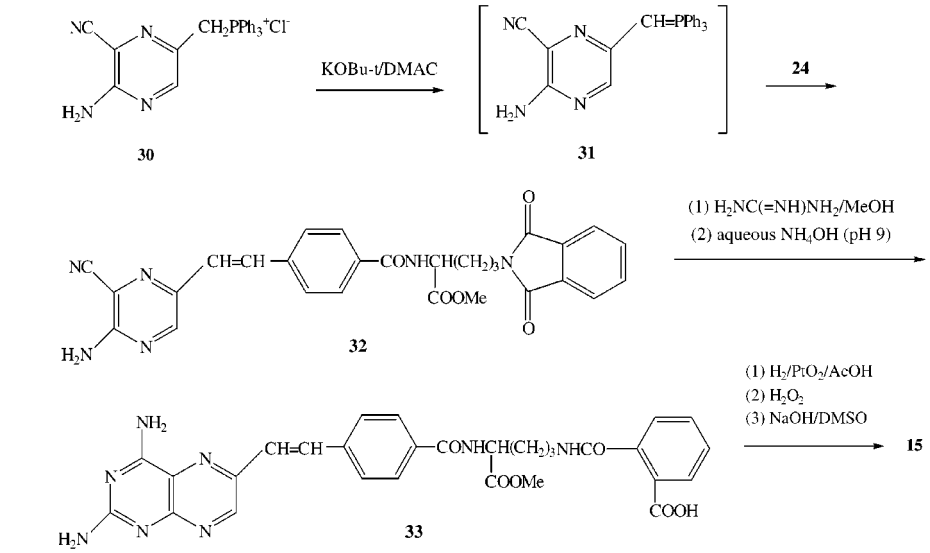
Three different approaches to the synthesis of 10-deaza analogues of **2** were investigated (Schemes 1−4). In general, these approaches are patterned after routes used previously to obtain the corresponding glutamate analogues.[20−25] Thus, as shown in Scheme 1, coupling of 4-formylbenzoic acid (**22**) to methyl 2-L-amino-5-phthalimidopentanoate hydrochloride (**23**·HCl)[17] via the mixed carboxylic anhydride (MCA) method afforded aldehyde **24**. The latter was used in a Wittig reaction with the putative 2,4-bis(phosphazene) ylide **25**,[26,27] arising either from pre-formed 2,4-diamino-6-(bromomethyl)pteridine hydrobromide (**26**·HBr)[28] or in situ from 2,4-diamino-6-(hydroxymethyl)pteridine (**27**)[29] as we described earlier.[30] In the present study, in contrast to others using **26**·HBr,[20,23] **25** was generated with potassium *tert*-butoxide. The ylide was readily detected by its dark-red color, which began to fade immediately upon addition of the aldehyde. The resulting olefin **28**, assumed (but not formally proved) to be a mixture of cis and trans isomers, was reduced catalytically to a mixture of 9,10-dihydro derivatives and the B-ring was reoxidized with H_2O_2 as described.[23] The overall yield of phthalimide ester **29** from **27** was 54%. Treatment of **29** with NaOH in warm MeOH for 1.75 h afforded **15** in 53% yield (29% overall). As in our previous experience with this type of chemistry, the phthalimide esters **28** and **29** were amenable to purification by chromatography on silica gel. When **29** was rigorously purified before final hydrolysis, **15** could be obtained in an analytically pure state without further chromatography.

An alternate route to **28** was also investigated (Scheme 2), involving a Wittig reaction between aldehyde **24** and the ylide generated from 2-amino-3-cyanopyrazin-5-ylmethyltriphenylphosphonium chloride (**30**).[31] To our knowledge, this was the first reported attempt to use **30** as an intermediate in the synthesis of 2,4-diamino-

Scheme 1



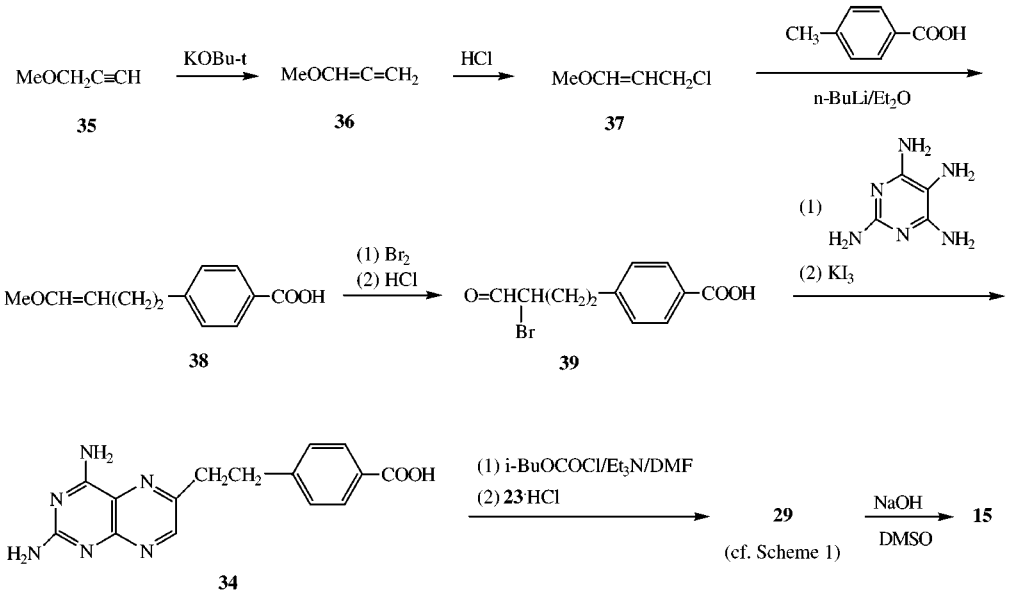
Scheme 2



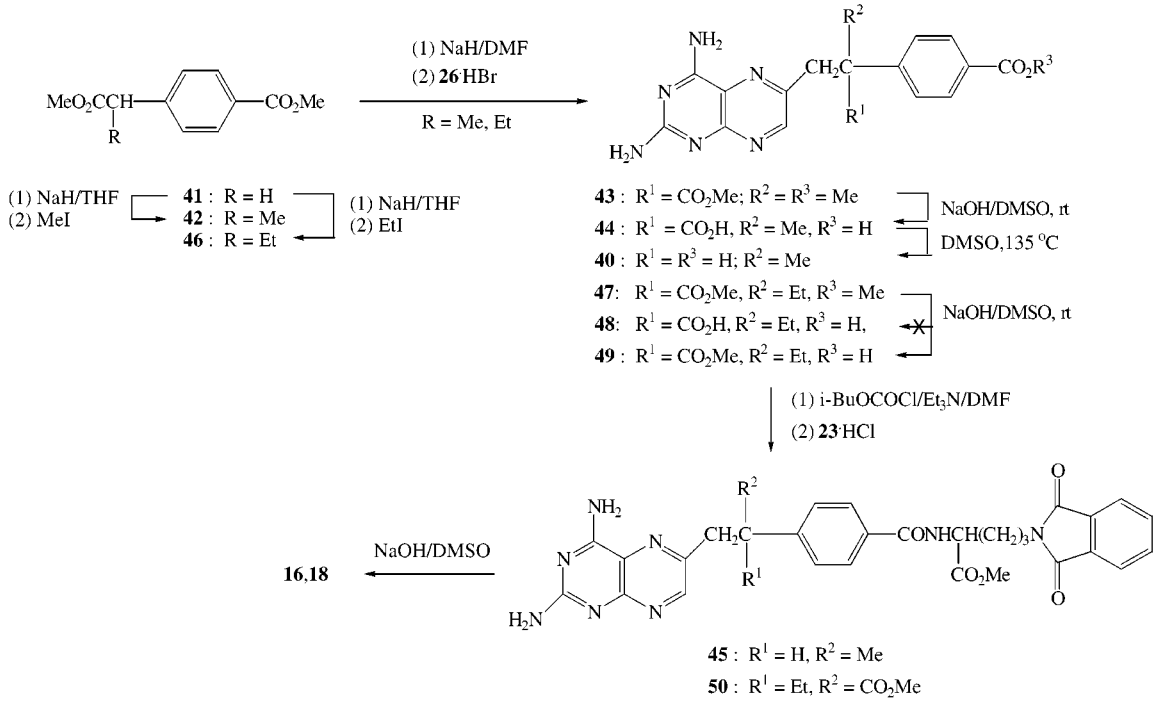
10-deazapteridines with an amino acid side chain. The dark-red ylide **31** formed very rapidly in the presence of potassium *tert*-butoxide,[32] and its reaction with the aldehyde appeared to proceed somewhat more rapidly than that of **25**. After flash chromatography on silica gel, olefin **32** was isolated in 40% yield. Like the pteridine **28** derived from ylide **25**, this product was assumed to be a mixture of the cis and trans olefins, but again the isomer ratio was not studied. Ring closure to a pteridine was performed by heating for ca. 4 days with guanidine in refluxing MeOH, and the product was purified by silica gel chromatography. Somewhat unexpectedly, microchemical analysis of the material isolated in this particular run suggested that it was not **28** but rather the monoammonium salt of the ring-opened structure **33**, whose catalytic reduction, reoxidation with H_2O_2, and treatment with NaOH afforded **15**. Cleavage of the phthalimide ring of **28** had apparently occurred when the product was dissolved and left to stand in aqueous ammonia at pH 9. Thus a way had been serendipitously found by which the pthalimide ring could be opened without cleaving the methyl ester. However, even though **15** could be made via pyrazine ylide **31**, this approach was judged to be less satisfactory than the one using pteridine ylide **25**.

The third route we utilized to obtain **15** (Scheme 3) was via MCA coupling of **23**·HCl with 4-amino-4-deoxy-10-deazapteroic acid (**34**). The latter was made from 2,4,5,6-tetraaminopyrimidine by an adaptation of the five-step sequence described in the literature.[21] Briefly,

Scheme 3



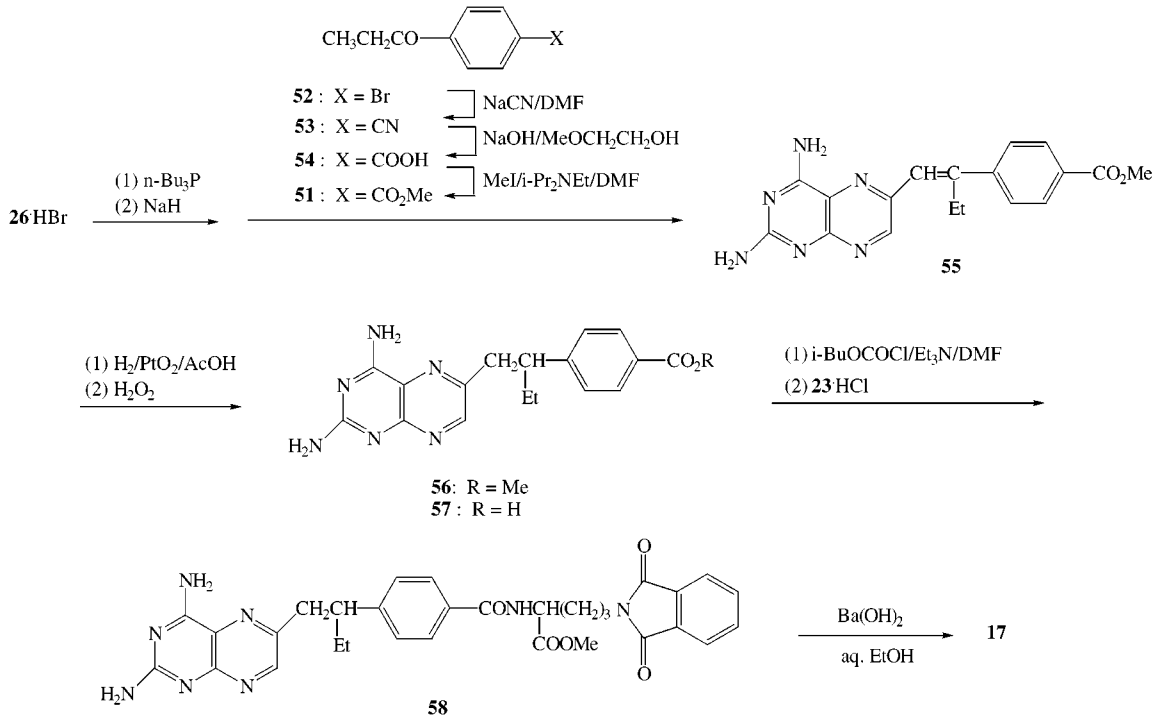
Scheme 4



3-methoxy-1-propyne (**35**) was converted sequentially to 1-methoxyallene (**36**) and 1-chloro-4-methoxy-2-butene (**37**), the latter was used to C-alkylate the dilithio derivative of 4-toluic acid, and the resulting enol ether **38** was subjected to bromination followed by acidolysis to obtain the key intermediate 2-bromo-4-(4-carboxyphenyl)butanal (**39**). The entire sequence was carried out in a continuous operation with each intermediate being used directly in the next reaction. The yield of **29** obtained from **39**, after chromatography, was 25%. Hydrolysis of **29** to **15** was carried out, in this case, with NaOH as the base and DMSO as the solvent. However, while it accelerated the reaction in comparison with MeOH (5 min versus 1.75 h), the use of DMSO did not substantially change the yield, which remained around 50−60%. Because the overall yield of **34** from *p*-toluic acid, in our hands, was <1%, we concluded that the more satisfactory route to **15** was via Scheme 1.

Having successfully used MCA coupling to prepare **15**, we also chose this method to obtain the 10-methyl-10-deaza analogue **16** (Scheme 4). The requisite intermediate 4-amino-4-deoxy-10-methyl-10-deazapteroic acid (**40**) was previously synthesized by DeGraw and coworkers[21] from **36** and the dilithio derivative of 4-ethylbenzoic acid, but in the present work **40** was prepared via a slight modification of the more recent method used by the same authors to obtain 10-propargyl-10-deazaaminopterin.[24] Although detailed procedures were published only for the 10-propargyl analogue, this method was described in a review article as being generally applicable to the synthesis of other 10-alkyl derivatives.[25] Briefly, dimethyl homotereph-

Scheme 5



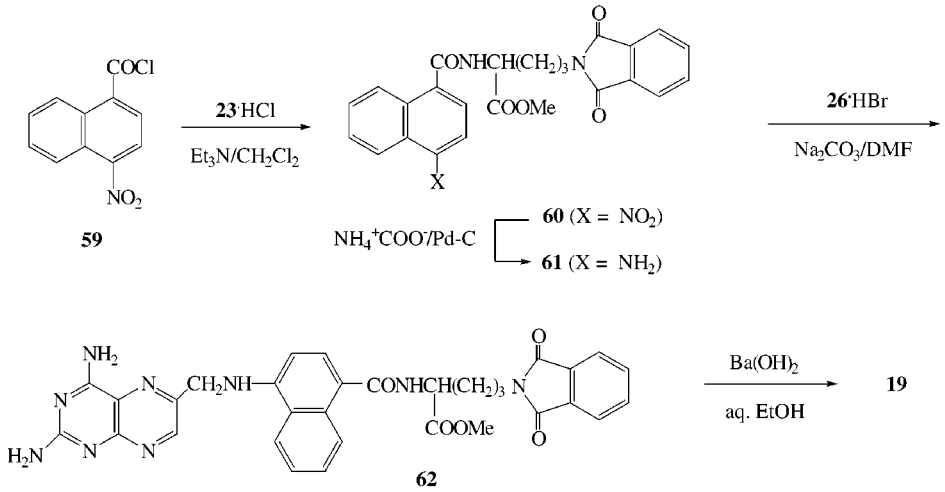
thalate (**41**) was C-alkylated with methyl iodide in the presence of NaH, and the resulting diester **42** (56% yield) was re-alkylated, this time with **26**·HBr, to obtain **43** (29% yield). After saponification to **44** (73% yield), selective removal of the benzylic carboxyl was accomplished in 59% yield by heating the diacid in DMSO at 135 °C. The overall four-step yield of **40** by this route was approximately 10%. Mixed anhydride coupling to **23**·HCl, followed by treatment with NaOH in DMSO, gave **45** (19%) and **16** (61%), respectively. As in the case of **15**, rigorous purification of the penultimate product **45** on a silica gel column allowed the final hemiphthaloyl derivative to be obtained in analytically pure state without further chromatography. The amount of **16** obtained via this sequence was again quite low, though it should noted that, since we desired only to prepare enough material for small-scale in vitro testing, the yield in each step of the process was not optimized. In agreement with the reported synthesis of the corresponding glutamate analogues,[21,24] **45** and **16** were isolated as unresolved 10*R*/10*S* mixtures. The presence of the two isomers was obvious from 500-MHz ^{1}H NMR spectra of these compounds, which featured two closely spaced singlets of equal intensity for the pteridine C-7 proton. However the C-7 proton in both **29** and **15** was a singlet.

An attempt was next made to synthesize the 10-ethyl analogue **17** by the same method as **16**. Thus, **41** was C-alkylated with ethyl iodide in the presence of NaH to obtain adduct **46** (Scheme 4). Further reaction with **26**·HBr afforded **47**, but when the latter compound was subjected to the saponification conditions we had used to prepare **40** (i.e., NaOH in DMSO at room temperature), the desired diacid **48** was not formed cleanly. This was confirmed by the ^{1}H NMR spectrum, which contained a sharp singlet at 3.6 ppm and no signal at 3.9 ppm, the latter being the chemical shift we had noted for the aromatic CO_2Me group in precursor **47**. Thus, it was evident that saponification had produced mainly **49** rather than **48**. Final confirmation of the structure of **49** came from the fact that MCA coupling with **23**·HCl, followed by the usual mild treatment with NaOH in DMSO, led to a product whose elemental analysis revealed a higher than expected C/N ratio and whose ^{1}H NMR spectrum still contained a sharp aliphatic ester singlet at δ 3.59. On the basis of these results we characterized the coupling product as **50** and the eventual hydrolysis product as **18**. To our knowledge, **18** is the first and only example of a 10-deaza antifolate with an ester group on C-10.

It should be noted that a number of experiments were done to see if hydrolysis of **47** would succeed under more vigorous conditions. Higher concentrations of NaOH, higher reaction temperatures, and solvents other than DMSO (e.g., refluxing 2-methoxyethanol) were tried, but to our surprise, the product always contained varying amounts of unreacted starting material. Moreover there were also more byproducts, possibly resulting from cleavage of the 4-amino group and rupture of the pyrimidine ring. Another method of ester cleavage involving the use of cyanide ion as a nucleophile was abandoned when it was found to lead to a complex mixture of products, possibly reflecting the known susceptibility of pteridines to cyanide attack at the C-7 position.[33] Other strong nucleophiles were not investigated.

Faced with this unexpected setback, we then decided to return to the Wittig approach as a route to **17**, and for this purpose needed to make a quantity of 4-(methoxycarbonyl)propiophenone (**51;** Scheme 5), which Piper and co-workers had used in their synthesis of edatrexate.[23] However, because we found in pilot experiments that the published routes to the carboxylic acid precursor of **51** from *p*-methylpropiophenone gave low and/or irreproducible yields for the oxidation of the methyl group with $KMnO_4$[34] or with O_2 in the presence of nickel

Scheme 6

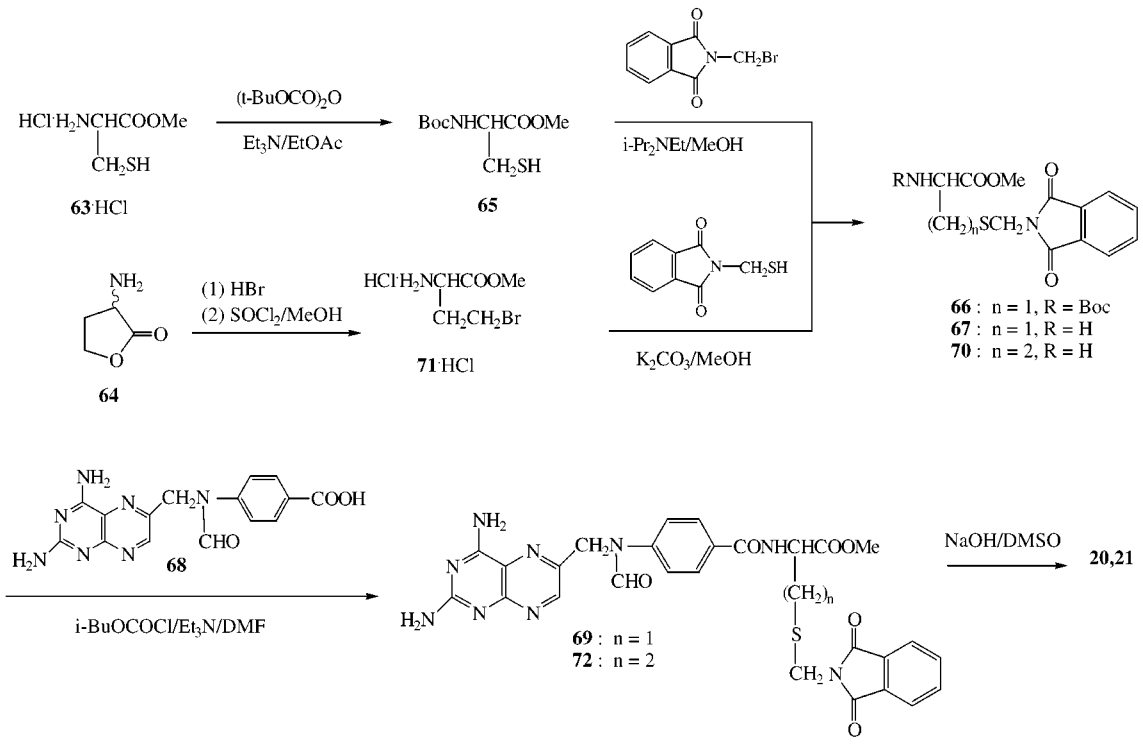


benzoate,[35] affording mainly terephthalic acid rather than 4-carboxypropiophenone, we developed a new and more reliable synthesis of **51** from the commercially available starting material 4-bromopropiophenone (**52**). As shown in Scheme 5, treatment of **52** with NaCN in DMF afforded the nitrile **53**, which on hydrolysis with NaOH in 2-methoxyethanol was converted to carboxylic acid **54**. Neutral esterification with MeI and *i*-Pr_2NEt in DMF completed the synthesis. The overall three-step yield of **51** by this route was 55%. Wittig condensation of **51** and **26**·HBr in the presence of *n*-Bu_3P, followed by catalytic hydrogenation and reoxidation with H_2O_2 as described,[23] yielded **55** and **56**, respectively. Several attempts to use Ph_3P in place of *n*-Bu_3P in the Wittig reaction were unsuccessful, probably because the ylide **25** is too sterically hindered to react with ketone **51**. Saponification of **56**, followed as usual by MCA coupling with **23**·HCl and brief treatment with $Ba(OH)_2$ in aqueous EtOH, afforded **57, 58**, and **17**, respectively.

The synthesis of the naphthyl analogue **19** is shown in Scheme 6 and was patterned after that of the corresponding glutamate.[27] Thus, 4-nitro-1-naphthoyl chloride (**59**) was prepared from 4-nitro-1,8-naphthoic anhydride and condensed with **23**·HCl to obtain the phthalimide ester **60** with an overall yield of 32%. Catalytic reduction of the nitro group, using ammonium formate as the H donor in the presence of 10% Pd−C, yielded the amine **61**, which was N-alkylated with **26**·HBr in the presence of Na_2CO_3 to obtain **62** (51% yield). Compounds **60** and **61** were purified by recrystallization and **62** by chromatography on silica gel. Hydrolysis of the methyl ester with concomitant opening of the phthalimide ring to form **19** (71% yield) was accomplished by stirring with $Ba(OH)_2$ in aqueous EtOH at room temperature for 24 h. A notable feature of the ^1H NMR spectrum of **19**, confirming the presence of the hemiphthaloyl group, was the chemical shift of the ornithine δ-CH_2, which was at 3.19 ppm as compared with 3.59 ppm in the ring-closed compound **62**. The same upfield displacement was likewise noted in the ^1H NMR spectra of the 10-deaza analogues **15**−**18** relative to their ring-closed precursors **29**, **45**, **50**, and **58** (see Experimental Section), thus proving that all these compounds are *dicarboxylic acids*. It is important that this be clearly established in every case, inasmuch as the very efficient active transport of the hemiphthaloyl-ornithine analogues into cells by the RFC is believed to reflect the presence in these compounds of two COOH groups, and the ring-closed form would have the same microchemical analysis as the ring-opened form with one less molecule of H_2O of solvation.

The cysteine and homocysteine derivatives **20** and **21** were synthesized as shown in Scheme 7, starting from commercially available methyl L-cysteinate hydrochloride (**63**·HCl) and D,L-2-aminobutyrolactone (**64**), respectively. The racemic lactone was used for reasons of economy, and it was assumed, by analogy with the well-known precedent of D- versus L-methotrexate,[36] that the product would have about one-half the activity of the pure L-enantiomer. Sequential reaction of **63**·HCl with di-*tert*-butyl dicarbonate in the presence of Et_3N afforded the Boc derivative **65** (61%), which on further reaction with *N*-bromomethylphthalimide in the presence of *i*-Pr_2NEt was converted to the phthalimide **66** (87%). Cleavage of the Boc group with *p*-TsOH in refluxing toluene afforded the tosylate salt **67**·TsOH (86%). The overall yield of **67**·TsOH from **63**·HCl was 46%. Coupling of **67**·TsOH to 4-amino-4-deoxy-N^{10}-formylpteroic acid (**68**) by the MCA method yielded the phthalimide ester **69**. The usual mild treatment with NaOH in DMSO then gave a mixture whose HPLC (C_{18} silica gel, 8% MeCN in 0.1 M NH_4OAc, pH 6.9) revealed two principal peaks in a ratio of ca. 2:1. Lyophilization of the major product and reprecipitation from dilute NH_4OH with AcOH afforded **20** with an overall yield 10% based on **67**·TsOH. The identity of the minor product was not studied. For the synthesis of **21**, the key intermediate methyl D,L-2-amino-5-[(2-phthalimidomethyl)thio]butanoate (**70**) was synthesized via a sequence recently used by us in another project.[37] Briefly, methyl D,L-2-amino-4-bromobutanoate hydrochloride (**71**·HCl) was prepared from **64** by acidolysis (HBr/AcOH) and esterification ($MeOH/SOCl_2$), and was condensed with *N*-mercaptomethylphthalimide in the presence of K_2CO_3 to obtain **70**. MCA coupling of **68** and **70** then gave the phthalimide **72**, As with other phthalimide esters described above, **72** was purified to homogeneity by silica gel column chromatography, whereas **21** was purified by reversed-phase preparative HPLC. The combined two-step yield of **21** from **70** was 19%. The presence of a hemiphthaloyl group in the final product was confirmed by its ^1H NMR spectrum, which

Scheme 7



revealed an upfield shift of ca. 0.3 ppm for the SCH_2N singlet in the ring-opened structure.

Biological Activity

The seven new analogues of **2** described in this paper were tested as inhibitors of the growth of cultured CCRF-CEM cells exposed continuously to drug for 72 h, a period of time that allows the nonpolyglutamatable analogues to be compared to classical DHFR inhibitors under conditions where the latter drugs have ample time to form polyglutamates in the cell. As in our previous work on second-generation analogues of **2**, the goal of this study was to compare the biological activities of these nonpolyglutamatable compounds with those of classical glutamate analogues, whose effect on cell growth is expected to reflect not only their influx and subsequent binding to DHFR but also their ability to form polyglutamates of different chain length and the affinity of these polyglutamates for enzymes other than DHFR. In contrast, inhibition of cell growth by nonpolyglutamatable analogues is assumed to be mechanistically more straightforward in that it probably reflects only influx and DHFR binding.

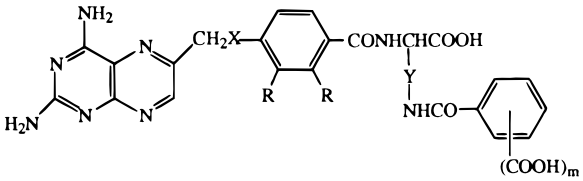
The IC_{50} values of **15**−**21** as inhibitors of CCRF-CEM cell growth are given in Table 1, along with comparison data for aminopterin, **2**, edatrexate, and four additional compounds (**3**−**7**) we had previously tested against other human tumor cell lines.[15,16] As may be seen from the results, decreasing the number of CH_2 groups in the amino acid side chain (**3**, **4**) and increasing this number (**5**) both gave a decrease in potency. Moving the COOH group to the meta and para positions (**6**, **7**) was likewise detrimental, particularly in the case of **7**, whose IC_{50} was 37-fold higher than that of **2**. These results demonstrated that the in vitro structure−activity patterns we had noted earlier[15,16] with these compounds using monolayer cultures of A549 non-small-cell lung and SCC25 head-and-neck squamous cell carcinoma can be extended to CCRF-CEM cells in suspension.

With regard to the new compounds reported here, replacement of N^{10} in **2** by CH_2 (**15**) or CHMe (**16**) led to a ca. 3-fold increase in potency. However, **17** was slightly less active than **15** or **16** and thus was not statistically different from **2**. The 10-ethyl-10-methoxycarbonyl analogue **18** had an IC_{50} of 54 ± 4.9 nM. Thus, this modification of the 10-position in **15** was clearly unfavorable.

It may be noted that 10-deazaaminopterin and edatrexate are reported[38] to be essentially like aminopterin in their growth inhibitory activity against Manca cells, which are likewise a human leukemic cell line but are of B-cell rather than T-cell lineage. The reported IC_{50} values for aminopterin and edatrexate against these cells are 1.4 ± 0.3 and 0.9 ± 0.2 nM, respectively, and thus are ca. 3-fold lower than our values for these compounds against CCRF-CEM cells. Whether this is due to a difference in sensitivity to DHFR inhibitors between B-cells and T-cells or to small differences in assay methodology is unknown. It is clear from our results, however, that the effect of replacing nitrogen by carbon at the 10-position on cell growth inhibition is qualitatively similar in the hemiphthaloylornithine derivatives as it is in glutamate derivatives even though only the latter can form polyglutamates.

Because of the chiral nature of C-10 in **16** and **17**, these compounds can exist as mixtures of diastereomers, as is also true for 10-methyl-10-deazaaminopterin and edatrexate.[22] Our 500-MHz ^1H NMR spectra indicate that both diastereomers are in fact present in **16** and **17** in roughly equal proportions, so that the IC_{50} values in Table 1 actually represent the averaged contributions of the 10*R* and 10*S* isomers. In the case of 10-methyl-10-deazaaminopterin and edatrexate, the 10*R* and 10*S* isomers have been synthesized in stereochemically pure

Table 1. Growth Inhibition of CCRF-CEM Human Leukemic Lymphoblasts by PT523 Analogues



compd	X	R	Y	m	IC_{50} (nM)[a]
AMT (**1**)	NH	H	*b*	−	4.4 ± 1.0 (0.34)
PT523 (**2**)	NH	H	$(CH_2)_3$	ortho	1.5 ± 0.39 (1)[c]
edatrexate	CHEt	H	*b*	−	3.3 ± 0.36 (0.45)
3	NH	H	CH_2	ortho	5.1 ± 0.58 (0.29)
4	NH	H	$(CH_2)_2$	ortho	5.5 ± 1.6 (0.27)
5	NH	H	$(CH_2)_3$	ortho	2.9 ± 0.97 (0.52)
6	NH	H	$(CH_2)_3$	meta	7.5 ± 0.47 (0.20)
7[d]	NH	H	$(CH_2)_3$	para	55 ± 2.0 (0.027)
15	CH_2	H	$(CH_2)_3$	ortho	0.53 ± 0.07 (2.8)
16	CHMe	H	$(CH_2)_3$	ortho	0.63 ± 0.08 (2.4)[e]
17[d]	CHEt	H	$(CH_2)_3$	ortho	1.2 ± 0.25 (1.3)[e]
18[d]	$C(Et)CO_2Me$	H	$(CH_2)_3$	ortho	54 ± 4.9 (0.028)[e]
19	NH	$(CH{=}CH)_2$	$(CH_2)_3$	ortho	1.2 ± 0.22 (1.3)
20	NH	H	CH_2SCH_2	ortho	4.4 ± 1.1 (0.34)
21[f]	NH	H	$(CH_2)_2SCH_2$	ortho	5.0 ± 0.56 (0.30)

[a] Cells were treated continuously with drug as described in the Experimental Section. Unless otherwise noted, each result is the mean ± SD ($n = 3$). Numbers in parentheses are normalized relative to PT523 (=1.0). [b] Glutamate side chain. [c] For **2**, $n = 10$. [d] For **7**, **17** and **18**, $n = 6$. [e] Tested as unresolved 10*R*,10*S* mixtures. [f] Tested as the unresolved D,L mixture.

form,[22] but the absolute configuration at C-10 was not established and the diastereomers of each compound were simply referred to as *l*,L and *d*,L isomers. However it was noted in growth inhibition assays against L1210 cells that the *d*,L isomers of 10-methyl-10-deazaaminopterin and edatrexate were 1.7- and 3.5-fold more potent than the respective *l*,L isomers. If we assume for the sake of simplicity that the *d*,L and *l*,L isomers of **16** and **17** are present in a 1:1 ratio and that their potencies differ by the same amount as those of the corresponding glutamates, a straightforward calculation leads to a predicted IC_{50} of 0.48 nM for *d*,L-**16** and 0.54 nM for *d*,L-**17** (i.e., essentially the same as the IC_{50} of **15**). However, since we do not actually know that the same differences in activity would be obtained for CCRF-CEM cells as for L1210, these values must be considered as merely estimates. While it would have been possible in principle to synthesize the *d*,L isomers of **16** and **17** from the *d*,L isomers of **39** and **55**, the laborious resolution of the latter compounds was not deemed worthwhile.

The IC_{50} of the naphthalene analogue **19** was found to be 1.2 ± 0.22 nM, a value 3.7-fold lower than that of **1** but not distinguishable from that of **2**. Although an IC_{50} for the corresponding glutamate analogue against CCRF-CEM cells is unknown, the IC_{50} of this compound against another human cell line, HL-60 promyelocytic leukemia, is reported to be 4.6 ± 1.0 nM as compared with 8.1 ± 1.0 nM for methotrexate.[38] Based on this indirect comparison, it appears that **19** may be a little more potent than its glutamate analogue. An interesting characteristic of the glutamate analogue that makes it particularly interesting is that it lacks detectable substrate activity for folylpolyglutamate synthetase from CCRF-CEM cells and thus can be viewed as a nonpolyglutamatable antifolate even though it contains a glutamate side chain. Its greater potency relative to methotrexate, like that of **2**, is probably due to improved transport and DHFR binding.

The sulfur analogues **20** and **21** were of interest to test because of their relationship to the previously synthesized analogues of **2** with CH_2, $(CH_2)_2$, and $(CH_2)_4$ side chains in place of $(CH_2)_3$.[15,16] It is a commonly held view that the S atom is bioisosterically equivalent to a CH=CH group (i.e., it is larger than a CH_2 group but smaller than a CH_2CH_2 group). On this basis we expected the side chain of **20** to be slightly shorter than that of the previously described lysine analogue **5**, whereas the side chain of **21** would be longer. This in turn would lead to altered distances between the α and aromatic COOH groups which could affect biological activity. As shown in Table 1, the IC_{50} of **20** was 4.4 ± 1.1 nM as compared with 2.9 ± 0.97 nM for **5** and 1.5 ± 0.39 nM for **2**, but unfortunately the difference between **20** and **5** was not large enough to allow us to draw any conclusion as to whether the small change in chain length embodied in structure **20** is significant in terms of cell growth inhibition. However it appeared from the results that **20** has about the same potency as aminopterin but is ca. 3-fold less potent than **2**. With respect to **21**, whose IC_{50} was 5.0 ± 0.56 nM, it may be noted that the sample tested was a D,L mixture, since the starting material was racemic. Since the IC_{50} values of L- and D-methotrexate against CCRF-CEM cells are reported to be 13 and >1000 nM,[36] respectively, it is reasonable to assume that the IC_{50} of the L form of **21** is as low as one-half the IC_{50} of the D,L mixture, or 2.5 nM. This would make it more potent than either **3** or **4**, as well as more potent than **20**, but still not as potent as **2**. We have previously noted that aminopterin analogues with nine and ten CH_2 groups in the side chain are more cytotoxic than the parent drug, at least against L1210 mouse leukemia.[39] Thus it is possible that similar elongation of the side chain of **2** to nine or more CH_2 groups would similarly have increased the potency of **2**.

In summary, the results in this study support the conclusion that, while the position of the phthaloyl

COOH group and the length of the amino acid side chain in **2** are important determinants of cytotoxic potency, changes in the pABA region and 9,10-bridge are well-tolerated and in some cases can even produce a several-fold increase in cytotoxic potency. Whether these increases are due to tighter DHFR binding, more efficient utilization of the RFC for influx into cells, or a combination of these factors is currently being investigated.

Experimental Section

IR spectra were obtained on a Perkin-Elmer model 281 double-beam spectrophotometer and UV spectra on a Varian model 210 instrument. For the sake of brevity, only IR peaks with wavenumbers greater than 1200 cm^{-1} are reported, and very weak peaks and shoulders are omitted. ^1H NMR spectra were recorded at 60 MHz with a Varian model EM360L instrument using Me_4Si as the reference or at 500 MHz with a Varian model ML500 instrument. The very broad amide NH signal in several of the intermediates (e.g., **24**) whose ^1H NMR spectrum was recorded at 60 MHz was indistinguishable from the baseline and thus is not reported. Analytical TLC was carried out on fluorescent Whatman MK6F silica gel-coated glass slides, and 254-nm UV illumination was used to visualize spots. Column chromatography was on Baker silica gel (regular grade, 60−200 mesh; flash grade, 40-μm particle size) or on Whatman DE-52 preswollen DEAE-cellulose. Moisture-sensitive reactions were carried out in solvents that were of Sure-Seal grade (Aldrich, Milwaukee, WI) or had been stored over Linde 4A molecular sieves. In the case of THF, the solvent was distilled from sodium benzophenone ketyl. HPLC separations were on C_{18} silica gel radial compression cartridges (Millipore, Milford, MA; analytical, 5-μm particle size, 5 × 100 mm; preparative, 15-μm particle size, 25 × 100 mm). Solids were generally dried over P_2O_5 at 50−80 °C in a vacuum oven or Abderhalden apparatus. Melting points (not corrected) were determined on a Fisher-Johns hot-stage microscope or in open Pyrex capillary tubes in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA). Methyl 2-L-amino-5-phthalimidopentanoate hydrochloride (**23**·HCl),[16] 2,4-diamino-6-(bromomethyl)pteridine hydrobromide (**26**·HBr),[27] 2,4-diamino-6-(hydroxymethyl)pteridine (**27**),[29] 4-amino-4-deoxy-N^{10}-formylpteroic acid (**68**),[3] and methyl *S*-(phthalimidomethyl)-D,L-homocysteinate hydrochloride (**70**·HCl)[37] were prepared as previously described. Starting materials **22**, **35** and **52** as well as other reagents and chemicals were purchased from Aldrich, Milwaukee, WI, or Fisher, Boston, MA. Microanalyses were performed by Robertson Laboratory, Madison, NJ, or Quantitative Technologies, Whitehouse, NJ, and were within ±0.4% of theory unless otherwise indicated.

Methyl 2-L-[*N*-(4-Formylbenzoyl)amino]-5-phthalimidopentanoate (24). *i*-BuOCOCl (262 mg, 1.93 mmol) was added to a stirred solution of **22** (289 mg, 1.93 mmol) and Et_3N (428 mg, 4.24 mmol) in dry DMF (10 mL). The reaction mixture was stirred at room temperature for 20 min and **23**·HCl (548 mg, 1.75 mmol) was added in portions. After another 30 min of being stirred at room temperature, the clear solution was evaporated to dryness. The residue was taken up in $CHCl_3$ and the solution extracted consecutively with 0.1 N HCl, H_2O, and saturated aqueous $NaHCO_3$, then dried ($MgSO_4$) and evaporated. The product was chromatographed on silica gel (flash grade, 55 g, 20.5 × 3 cm) with $CHCl_3$ as the eluent. Fractions giving a single TLC spot with R_f 0.53 (silica gel, 95:5 $CHCl_3$−MeOH) were pooled and evaporated to obtain **24** as colorless crystals (400 mg, 56%): mp 166−167 °C; IR (KBr) ν 3460, 3320, 2950, 2850, 1770, 1745, 1690−1710, 1680, 1640, 1570, 1530, 1500, 1460, 1450, 1430, 1400, 1380, 1350, 1330, 1300 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.6−2.3 (m, 4H, β- and γ-CH_2), 3.75 (s, 5H, OMe and δ-CH_2), 4.8 (m, 1H, α-CH), 7.7−7.9 (complex m, 8H, aromatic protons), 10.1 (s, 1H, CH═O). Anal. ($C_{22}H_{20}N_2O_6$) C, H, N.

Methyl 2-L-[4-[2-(2-Amino-3-cyanopyrazin-2-yl)vinyl]-benzoyl]amino-5-phthalimidopentanoate (32). A solution of *t*-BuOK in DMAC was prepared by adding KH (35% w/w in mineral oil; 105 mg, calculated to contain 36 mg, 0.9 mmol) in *t*-BuOH, evaporating the solution to dryness, and taking up the residue in dry DMAC (5 mL). The slightly cloudy solution was added to a stirred suspension of phosphonium salt **30** (215 mg, 0.5 mmol)[31] in dry DMAC (5 mL) at room temperature. The intense red color of the ylide formed immediately and lightened upon addition of the aldehyde **24** (207 mg, 0.5 mmol). The reaction mixture was stirred at room temperature for 26 h, the solvent was removed by rotary evaporation (vacuum pump, receiver cooled in dry ice/acetone), and the residue was redissolved in $CHCl_3$ (100 mL). The solution was washed with H_2O (2 × 50 mL), dried ($MgSO_4$), concentrated to a small volume, and chromatographed on flash-grade silica gel (11 g, 1.5 × 12 cm) with 98:2 $CHCl_3$−MeOH as the eluent. Homogeneous fractions giving a yellow TLC spot with R_f 0.29 (silica gel, 95:5 $CHCl_3$−MeOH) were pooled and evaporated to obtain **32** as a yellow solid (106 mg, 40%). Recrystallization from a mixture of CH_2Cl_2 and Et_2O afforded light-yellow crystals: mp 116−117 °C; IR (KBr) ν 3420, 3315, 3220, 2940, 2215, 1770, 1745, 1640, 1610, 1565, 1520, 1490, 1360 cm^{-1}; UV λ_{max} (95% EtOH) 233, 242, 326 nm; ^1H NMR ($CDCl_3$) δ 1.7−2.2 (m, 4H, β- and γ-CH_2), 3.75 (t, 2H, δ-CH_2), 3.76 (s, 3H, OMe), 4.87 (m, 1H, α-CH), 5.25 (s, 2H, NH_2), 6.84 (d, 1H, vinyl H), 7.07 (d, 1H, vinyl H), 7.5−7.9 (m, 8H, aromatic), 8.27 (s, 1H, pyrazine 6-H). Anal. ($C_{18}H_{24}N_6O_5$·$0.4H_2O$) C, H, N.

Methyl 2-L-[4-[2-(2,4-Diaminopteridin-6-yl)vinyl]benzoyl]amino-5-phthalimidopentanoate (28). Br_2 (240 mg, 1.5 mmol) was added dropwise to a stirred solution of Ph_3P (393 mg, 1.5 mmol) in DMAC (5.5 mL, dried over molecular sieves) while keeping the temperature at 0−5 °C. After 5 min, 2,4-diamino-6-hydroxymethylpteridine (**27**) (97 mg, 0.5 mmol) was added and stirring was continued at room temperature for 20 h. Additional DMAC (4 mL) and Ph_3P (138 mg, 0.53 mmol) were then added and the solution was heated at 64−66 °C for 2 h. In a separate flask, a solution of *t*-BuOK was prepared in *t*-BuOH (3 mL) by adding KH (35% w/w in mineral oil, 458 mg; calculated to contain 160 mg, 4 mmol), and when H_2 evolution ceased the alcohol was evaporated under reduced pressure and replaced with dry DMAC (3 mL). The contents of the first flask were added to the second flask with the help of an additional small volume of DMAC to effect transfer. Aldehyde **24** (207 mg, 0.5 mmol) was then added, whereupon partial decolorization occurred. After 15 min the reaction mixture was diluted with dry THF (5 mL), stirring at room temperature was continued for 3.5 days, and the reaction was quenched with glacial AcOH (0.5 mL). The dark reaction mixture was filtered, the filter cake was washed with MeOH, and the combined filtrate and wash solution were evaporated to dryness under reduced pressure. The residue was dispersed in benzene (10 mL) by sonication and was filtered. The solid was triturated with $CHCl_3$ (75 mL), and the $CHCl_3$ extract was decanted from an insoluble black tar and washed with H_2O (75 mL). The aqueous layer was re-extracted with $CHCl_3$ (50 mL), and the combined $CHCl_3$ extracts were washed again with H_2O (75 mL), dried over $MgSO_4$, and evaporated. The material which formed at the $CHCl_3$−H_2O interface was collected and dried in vacuo at 60 °C over P_2O_5 to obtain a pale-yellow solid (58 mg) whose TLC contained a strong yellow spot (R_f 0.87, silica gel, 28:12:1 $CHCl_3$−MeOH−28% NH_4OH). The residue after evaporation of the $CHCl_3$ layer was taken up by sonication in warm 28:12:1 $CHCl_3$−MeOH−28% NH_4-OH. A total of 25 mL was required to dissolve the sample. The solution was applied onto a column of silica gel (flash grade, 20 g, 22 × 1.5 cm), which had been prepared with 85:15:1 $CHCl_3$−MeOH−28% NH_4OH and was eluted with the same solvent. Homogeneous fractions (TLC: R_f 0.25, silica gel 9:1 $CHCl_3$−MeOH) were pooled and evaporated to obtain **28** as a pale-yellow solid (102 mg), thus bringing the total yield to 160 mg (54%). A sample for microanalysis, assumed to contain cis and trans isomers, was obtained by recrystallization from a $CHCl_3$−Me_2CO−Et_2O mixture and melted at 148−155 °C: IR (KBr) ν 3380, 3200, 2950, 1770, 1740, 1710, 1640−1620 br, 1580, 1560, 1500, 1440, 1400, 1365, 1310 cm^{-1}; ^1H NMR

(DMSO-d_6) δ 1.6−1.9 (m, 4H, δ- and γ-CH_2), 3.61 (m, 5H, OMe and δ-CH_2), 4.45 (m, 1H, α-CH), 6.82 (s, 2H, NH_2), 6.6−8.5 (complex m, 10H, vinyl and aromatic H), 8.73 (d, 0.33H, 7-H of one vinyl isomer), 8.85 (s, 0.66H, 7-H of other vinyl isomer). Anal. ($C_{19}H_{26}N_9O_6 \cdot 1.6H_2O$) C, H, N.

Methyl N^{α}-[4-[2-(2,4-Diaminopteridin-6-yl)vinyl]benzoyl]-N^{δ}-hemiphthaloyl-L-ornithinate (33). Metallic Na (14 mg, 0.6 mmol) was added to MeOH (2.5 mL) and the solution was added to a solution of guanidine hydrochloride (57 mg, 0.6 mmol) in MeOH (15 mL). Amino nitrile **32** (106 mg, 0.2 mmol) was then added, and after being stirred under reflux for 43 h the mixture was poured into H_2O (100 mL). The solution was adjusted to pH 3.9 with 10% AcOH and left at 5 °C for 2 days. The precipitate was collected by centrifugation, washed twice with H_2O, and dried on a lyophilizer to obtain a yellow powder (115 mg). The crude product was chromatographed on flash-grade silica gel (5 g, 1.0 × 15.5 cm) with 5:4:1 $CHCl_3$−MeOH−28% NH_4OH as the eluent, and homogeneous fractions (TLC: R_f 0.56, silica, 5:4:1 $CHCl_3$−MeOH−28% NH_4OH) were pooled and evaporated. The residue was redissolved in dilute ammonia at pH 9−10, and the pH was adjusted to 4.3 with 10% AcOH to obtain a gelatinous orange precipitate which became more granular after the acidified mixture was kept overnight at 5 °C. The solid was collected by centrifugation, washed with H_2O, and dried on a lyophilizer to obtain a yellow solid (45 mg, 33%) whose elemental analysis was consistent with a hydrated *monoammonium salt* of **33**: mp 210−214 °C; IR (KBr) ν 3400 br, 2925, 1630, 1550, 1530, 1500, 1440, 1370, 1300, 1270−1240 cm^{-1}; UV λ_{max} (95% EtOH) 323, 334 infl, 406 nm. Anal. ($C_{29}H_{28}N_8O_6 \cdot NH_3 \cdot 4H_2O$) C, H, N.

For further elaboration to **15**, this material was combined with **28** and subjected to catalytic hydrogenation, reoxidation, and alkaline hydrolysis as described for the synthesis of **15** via the ring-closed intermediate **29** (vide infra).

4-[2-(2,4-Diaminopteridin-6-yl)ethyl]benzoic Acid (34). Step 1. 3-Methoxypropyne (**35**; 20 g, 0.28 mol) was added to *t*-BuOK (0.9 g, 0.008 mol) which had been dried in vacuo at 60 °C over P_2O_5. The mixture was refluxed under N_2 at 70 °C for 4 h, and allene **36** (17 g, 83%) was isolated by short-path distillation into a receiver cooled in dry ice. A solution of gaseous HCl (2.7 g, 0.074 mol) in dry Et_2O (45 mL) was added dropwise under N_2 to a solution of **36** (5.2 g, 0.074 mol) in dry Et_2O (26 mL) at −78 °C. The reaction mixture was stirred at −78 °C for 30 min, and the resulting solution of **37** was kept at 0−5 °C for 40 h until it could be used in the next step.

Step 2. A solution of *n*-BuLi in hexanes (87.5 mL, calculated to contain 0.14 mol) was added dropwise under N_2 to a stirred solution of *i*-Pr_2NH (14 g, 0.14 mol, freshly distilled from NaH) in dry THF (205 mL) while maintaining the temperature at 0−5 °C. After 30 min, a solution of *p*-toluic acid (9.5 g, 0.07 mol) in dry THF (40 mL) was added. The dark-red mixture was stirred at 0−5 °C for 3.5 h and after being kept in the refrigerator for 22 h was treated dropwise at 0−5 °C under N_2 with the solution of **37** prepared above. The red color faded gradually over 2 h, whereupon the solvents were evaporated and the residue was partitioned between Et_2O (150 mL) and H_2O (200 mL). The aqueous layer was chilled and adjusted to pH 8−9 with dry ice. To the resulting solution of enol ether **38** were then added CH_2Cl_2 (200 mL), followed dropwise by a 1 M solution of Br_2 in CH_2Cl_2 while maintaining a pH of 7−8 with solid $NaHCO_3$. Addition was continued until persistence of the red color of Br_2 was noted, whereupon the mixture was acidified to pH 2 with 6 N HCl, and the organic layer was separated. The aqueous layer was washed with CH_2Cl_2, and the combined organic layers were dried ($MgSO_4$) and evaporated to a semisolid (13 g, 61%) whose ^{1}H NMR showed it to be a 62:38 mixture of the desired α-bromo aldehyde **39** and unchanged p-toluic acid. The mixture was used in the next step without further purification.

Step 3. A mixture of 2,4,5,6-tetraaminopyrimidine sulfate (7.2 g, 0.03 mol) and $BaCl_2 \cdot 2H_2O$ (7.3 g, 0.03 mol) in H_2O (145 mL) was stirred at room temperature for 1.5 h, then warmed to 70 °C in a water bath, and filtered while hot. The filtrate was cooled to room temperature, adjusted to pH 3−5 with 10% NaOH, warmed back to 50 °C, and treated dropwise with a solution of crude **39** (calculated to contain 8.5 g, 0.03 mol) in AcOH (50 mL). An insoluble gum formed during the reaction. Stirring at 50 °C was continued for 1.5 h, and the supernatant was poured off from the gum, left to cool to room temperature, and treated with small portions of freshly prepared 5 N KI_3 solution until no more color change was seen. The mixture was then left in the refrigerator for 24 h, and the solid was collected, washed with H_2O and Et_2O, and stirred for 2 h in H_2O (60 mL) containing 28% NH_4OH (1.5 mL). Filtration and acidification of the filtrate with 10% AcOH led to precipitation of **34** as a yellow solid, which was collected, washed with H_2O, and dried; yield 0.23 g (1% from *p*-toluic acid). This material was used directly for subsequent coupling to **23**·HCl (vide infra).

Methyl 2-L-[4-[2-(2,4-Diaminopteridin-6-yl)ethyl]benzoyl]amino-5-phthalimidopentanoate (29). Method A. A solution of **28** (150 mg, 0.25 mmol) in glacial AcOH (30 mL) was shaken in the presence of PtO_2 (30 mg) under H_2 (initial pressure 15 lb/in.^{2}) for 19 h. After filtration of the catalyst through Celite, the solvent was evaporated to dryness with the help of EtOH to coevaporate the last traces of entrapped AcOH. The residue was taken up in a mixture of absolute EtOH (40 mL) and 1 N HCl (1 mL), and the solution was treated with 30% H_2O_2 (0.5 mL) and left in an open flask for 1.75 h. Excess Et_3N was added, the solution was evaporated to dryness under reduced pressure, the residue was taken up in a mixture of absolute EtOH and $CHCl_3$, the solution was evaporated again, and the final residue was dissolved in a small volume of 95:5 $CHCl_3$−MeOH and applied onto a column of flash grade silica gel (6 g, 15 × 1.0 cm) which was prepared and eluted with the same solvent mixture. Homogeneous fractions (TLC: R_f 0.32, blue-fluorescent spot, 9:1 $CHCl_3$−MeOH) were pooled and evaporated to a yellow powder **29** (124 mg, 81%). The analytical sample, prepared by recrystallization of a small portion of this solid from a mixture of MeOH and *i*-PrOH, melted at 162 °C dec (softening above 131 °C): IR (KBr) ν 3410, 2930, 2940, 2740, 2680, 2500, 1730, 1710, 1635, 1560−1540, 1500, 1480, 1490, 1400 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.66−1.78 (m, 4H, β- and γ-CH_2), 3.2−3.3 (m, 4H, bridge CH_2CH_2), 3.59 (s, 5H, δ-CH_2 and COOMe), 4.4 (m, 1H, α-CH), 6.2 (br s, 2H, NH_2), 7.3 (m, 2H, 3′- and 5′-H), 7.6 (bs s, 2H, NH_2), 7.7 (m, 2H, 2′- and 6′-H), 7.8 (m, 4H, phthaloyl ring protons), 8.52 (s, 1H, pteridine 7-H), 8.6 (d, 1H, CONH). Anal. ($C_{29}H_{28}N_8O_5 \cdot 2H_2O$) C, H, N.

Method B. A mixture of **34** (270 mg, 0.87 mmol) and Et_3N (175 mg, 1.74 mmol) in dry DMF (15 mL) was stirred at 80 °C until all the solid dissolved. The solution was cooled to 0−5 °C, treated dropwise with *i*-BuOCOCl (238 mg, 1.74 mmol) and stirred for 1.5 h. To the solution were then added **23**·HCl (540 mg, 1.74 mg) and Et_3N (175 mg, 1.74 mmol). Stirring was continued at 0−5 °C for 2 h, then at room temperature for 24 h, and the reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in a minimum of 9:1 $CHCl_3$−MeOH, and the solution was applied onto a column of flash-grade silica gel (20 g, 2 × 20 cm), which was eluted with 97:3 $CHCl_3$−MeOH. Fractions giving a single TLC spot with R_f 0.32 (silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated, the residue was redissolved in a minimal volume of 9:1 $CHCl_3$−MeOH, and the solution was added dropwise to excess ether. The precipitate was filtered, washed with Et_2O, and dried at room temperature in vacuo to obtain **29** as a yellow powder (119 mg, 25%). Melting points and IR and ^{1}H NMR spectra of the products obtained by methods A and B were indistinguishable and are given above.

N^{α}-[4-[2-(2,4-Diaminopteridin-6-yl)ethyl]benzoyl]-N^{δ}-hemiphthaloyl-L-ornithine (15). Method A. A solution of **29** (44 mg, 0.072 mmol) in warm MeOH (30 mL) was cooled to room temperature, treated with 1 N NaOH (0.75 mL), and stirred for 1.75 h. The pH was adjusted to pH 5.2 with 10% AcOH, and the mixture was left at 5 °C for 1 h and filtered. The solid was washed with H_2O (2 mL) and dried on a lyophilizer to obtain **15** as a pale-yellow solid (24 mg, 53%): mp >210 °C dec (darkening above 175 °C); HPLC 15 min (C_{18}

silica gel, 10% MeCN in 0.1 M NH_4OAc, pH 6.8, 1 mL/min); IR (KBr) ν 3400 br, 2930, 2860, 1645 br, 1565−1535, 1505, 1450, 1400 cm^{-1}; UV λ_{max} (pH 7.4) 230 infl (ϵ 23200), 255 (29500), 280 infl (5700), 370 (7100) nm; ^1H NMR (DMSO-d_6) δ 1.4−1.6 (m, 4H, β- and γ-CH_2), 2.8−3.2 (m, 6H, CH_2CH_2 bridge and δ-CH_2), 4.36 (m, 1H, α-CH), 6.55 (m, 2H, NH_2, exchangeable with D_2O), 7.32 (m, 2H, 3′- and 5′-H), 7.34−7.60 (m, 4H, phthaloyl H), 7.6 (m, 2H, NH_2, exchangeable with D_2O), 7.77 (m, 2H, 2′- and 6′-H), 8.28 (t, 1H, phthaloyl CONH, exchangeable with D_2O), 8.46 (d, 1H, benzoyl CONH), 8.49 (s, 1H, pteridine 7-H).

Method B. A solution of **29** (50 mg, 0.09 mmol) in DMSO (0.5 mL) was treated with 2 N NaOH (0.13 mL) for 5 min at room temperature. After exactly 5 min, the solution was diluted with 7.5 mL of H_2O, cooled to 5 °C, and adjusted to pH 4.3 with 10% AcOH. The precipitated solid was filtered, washed with H_2O, and dried in vacuo at 50 °C to obtain **15** as a pale-yellow solid (30 mg, 58%): mp >220 °C, darkening above 175 °C. Melting points and IR and ^1H NMR spectra of the product obtained via methods A and B were indistinguishable and are listed above. Anal. ($C_{28}H_{28}N_8O_6$·0.5CH_3COOH·0.9H_2O) C, H, N.

Methyl 2-L-[4-[2-(2,4-Diaminopteridin-6-yl)propyl]benzoyl]amino-5-phthalimidopentanoate (45). Step 1. Dimethyl homoterephthalate (**41**) (6 g, 28.8 mmol) was added to a suspension of NaH (60% w/w in mineral oil; 1.27 g, calculated to contain 762 mg, 31.7 mmol) in dry THF (150 mL) at 0 °C. After 1 h of stirring, MeI (4.5 g, 31.7 mmol) was added, and stirring was continued at 0 °C for 30 min and then at room temperature for 16 h. The reaction mixture was treated with 50% AcOH (2.9 mL) and poured into H_2O (300 mL). The product was extracted into Et_2O (3 × 100 mL), the combined extracts were dried ($MgSO_4$) and evaporated, and the residue was purified by flash chromatography on silica gel. The column was packed with 95:5 hexanes−EtOAc and then eluted sequentially with 95:5, 94:6, and finally 93:7 hexanes−EtOAc. Fractions giving a single TLC spot (R_f 0.33, silica gel, 92:8 hexanes−EtOAc) were pooled and concentrated to obtain **42** as a colorless oil which was used directly in the next reaction (3.56 g, 56%): ^1H NMR ($CDCl_3$) δ 1.55 (d, 3H, CH*Me*), 3.6 (m, 4H, COOMe and *CH*Me), 3.9 (s, 3H, aromatic COOMe), 7.5−8.1 (m, 4H, aromatic protons).

Step 2. A solution of diester **42** (2.40 g, 10.8 mmol) in dry DMF (10 mL) was added dropwise to a stirred suspension of NaH (60% w/w in mineral oil; calculated to contain 259 mg, 10.8 mmol) in dry DMF (10 mL) precooled to −5 °C. The reaction mixture was stirred at 0 °C for 5 min, then cooled to −35 °C, and treated dropwise with a solution of **26**·HBr (1.4 g, 3.6 mmol) in dry DMF (20 mL), while keeping the temperature between −45 and −35 °C during addition. The temperature was allowed to rise to 10 °C and stirring was continued for 2.5 h. The pH was adjusted to 7.0 with dry ice, and the DMF was removed by rotary evaporation (vacuum pump, dry ice/acetone). The residue was washed with EtOAc and H_2O and dried in vacuo at 60 °C to obtain **43** as a yellowish-brown powder (1.22 g, 29%) which was used without further purification: mp 170 °C, darkening above 155 °C; IR (KBr) ν 3450, 3310, 3150, 2950, 1720, 1625, 1560, 1440, 1280 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.5 (s, 3H, CH*Me*), 3.6 (m, 5H, CH_2 and benzylic COOMe), 3.8 (s, 3H, aromatic COOMe), 7.5−8.8 (m, 4H, aromatic protons), 8.35 (s, 1H, pteridine 7-H).

Step 3. Diester **43** (1 g, 2.5 mmol) was dissolved in DMSO (5 mL) with slight warming and 5 N NaOH (5 mL, 25 mmol) was added. The reaction was stirred at room temperature for 5 min, diluted with H_2O (50 mL), and adjusted to pH 4.0 with 10% AcOH while cooling in an ice bath. The precipitate was filtered, washed with H_2O, and dried in vacuo at 60 °C to obtain **44** as a yellow powder (670 mg, 73%): mp >220 °C dec; IR (KBr) ν 3350, 2950, 1640, 1560, 1380, 1250 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.6 (s, 3H, CH*Me*), 3.6 (m, 2H, CH_2), 6.5 (br s, 2H, NH_2), 7.5−8.0 (m, 4H, aromatic protons), 8.4 (s, 1H, pteridine 7-H).

Step 4. A portion of the foregoing solid (30 mg, 0.08 mmol) was dissolved in DMSO (0.2 mL) and the mixture was immersed in an oil bath preheated to 135 °C. The flask was fitted with a reflux condenser and kept in the bath while the progress of the reaction was monitored by HPLC (C_{18} silica gel, 25% MeCN in 0.1 M NH_4OAc, pH 7.5, 1 mL/min). The peak eluting at 8 min, corresponding to **43**, was gradually replaced by new peak eluting at 18 min. After 6 h the reaction was stopped and the solution was cooled to room temperature and diluted with H_2O. The precipitate was fitered, washed with H_2O, and redissolved in 10% NH_4OH. The pH was adjusted to 4.0 with 10% AcOH while cooling in ice, and the precipitate was collected, washed with H_2O, and dried in vacuo at 60 °C to obtain **40** as a yellow powder (15 mg, 57%) which was combined with another larger batch and used directly in the next reaction: mp >200 °C dec; IR (KBr) ν 3450, 3200, 2960, 1630, 1555, 1450, 1375, 1260 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.24 (d, 3H, CH*Me*), 3.1 (m, 2H, CH_2), 3.4 (m, 1H, *CH*Me), 6.5 (br s, 2H, NH_2), 7.3 (m, 2H, 3′- and 5′-H), 7.8 (m, 2H, 2′- and 6′-H), 8.4 (s, 1H, pteridine 7-H).

Step 5. A mixture of **40** (296 mg, 0.91 mmol) and Et_3N (184 mg, 1.82 mmol) was dissolved in dry DMF (15 mL) with slight warming. The solution was cooled to 0−5 °C, *i*-BuOCOCl (249 mg, 1.82 mmol) was added, and the reaction mixture was stirred for 1.5 h. Phthalimide **23**·HCl (567 mg, 1.82 mmol) was then added, followed by another portion of Et_3N (184 mg, 1.82 mmol). After another 2 h at 0−5 °C, the reaction mixture was stirred at room temperature for 24 h and concentrated to dryness by rotary evaporation (vacuum pump, receiver cooled in dry ice/acetone). The residue was dissolved in $CHCl_3$, the solution washed with H_2O, and the organic layer dried ($MgSO_4$) and chromatographed on silica gel (20 g, 2 × 20 cm) with 98:2 followed by 97:3 $CHCl_3$−MeOH as the eluents. Fractions giving a single yellow spot with R_f 0.37 (silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated to dryness, the residue was taken up in a minimal volume of 9:1 $CHCl_3$−MeOH, and the solution was added to excess Et_2O. The precipitated solid was filtered, washed with Et_2O, and dried in vacuo at 60 °C to obtain **45** as a yellow powder (100 mg, 19%): mp 135 °C, darkening above 115 °C; IR (KBr) ν 3420, 2950, 1710, 1620, 1560, 1540, 1500, 1440, 1400, 1350 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.25 (m, 3H, CH*Me*), 1.60−1.77 (m, 4H, β- and γ-CH_2), 3.08 (m, 2H, 9-CH_2), 3.50 (m, 1H, C*H*Me), 3.57 (m, 5H, δ-CH_2 and OMe), 4.40 (m, 1H, α-CH), 6.5 (br s, 2H, NH_2), 7.33 (m, 2H, 3′- and 5′-H), 7.77 (m, 2H, 2′- and 6′-H), 7.82−7.84 (m, 4H, phthaloyl ring protons), 8.30 (d, 1H, pteridine 7-H), 8.57 (d, 1H, CONH). Anal. ($C_{30}H_{30}N_8O_5$·H_2O) C, H, N.

***N*$^{\alpha}$-[4-[2-(2,4-Diaminopteridin-6-yl)propyl]benzoyl]-*N*$^{\delta}$-hemiphthaloyl-L-ornithine (16).** Ester **45** (50 mg, 0.086 mmol) was dissolved in DMSO (0.5 mL), 1 N NaOH (0.26 mL, 0.26 mmol) was added, and the reaction mixture was stirred at room temperature for exactly 5 min and then diluted with H_2O (8 mL). The pH was adjusted to 4.0 with 10% AcOH, and the precipitate was filtered, washed with H_2O, and dried in vacuo at 60 °C to obtain **16** as a pale-yellow powder (31 mg, 61%): mp >190 °C dec; HPLC 15 min (C_{18} silica gel, 0.1 M NH_4OAc, pH 6.8, 1 mL/min); IR (KBr) ν 3420, 2950, 1640, 1550, 1500, 1450, 1400, 1360, 1300 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.25 (d, 3H, CH*Me*), 1.56 (m, 2H, β-CH_2), 1.8 (m, 2H, γ-CH_2), 3.08 (m, 2H, 9-CH_2), 3.19 (m, 2H, δ-CH_2), 3.47 (m, 1H, C*H*Me), 4.35 (m, 1H, α-CH), 6.6 (br s, 2H, NH_2), 7.33 (m, 2H, 3′- and 5′-H), 7.47−7.76 (m, 5H, phthaloyl ring protons and phthaloyl CONH), 7.77 (m, 2H, 2′- and 6′-H), 8.37 (d, 1H, pteridine 7-H), 8.4 (d, 1H, CONH). Anal. ($C_{29}H_{30}N_8O_6$·H_2O) C, H, N.

Methyl 2-L-[4-[1-(2,4-Diaminopteridin-6-yl)-2-(methoxycarbonyl)-2-butyl]benzoyl]amino-5-phthalimidopentanoate (50). Step 1. Dimethyl homoterephthalate (**41**; 9 g, 43 mmol) was added to a suspension of NaH (60% w/w in mineral oil; 3.2 g, calculated to contain 1.9 g, 48 mmol) in dry THF (225 mL) at 0 °C. After 1 h of stirring, ethyl iodide (7.4 g, 48 mmol) was added, and stirring was continued at 0 °C for 1 h and then at room temperature for 16 h. The reaction mixture was treated with 50% AcOH (4.4 mL) and poured into H_2O (300 mL). The product was extracted into Et_2O (3 × 125 mL), the combined extracts were dried ($MgSO_4$) and evapo-

rated, and the residue was purified on a silica gel column packed with 95:5 heptane−EtOAc and eluted with 95:5, 94:6, and finally 93:7 heptane−EtOAc. Fractions giving a single TLC spot (R_f 0.25, silica gel, 92:8 heptane−EtOAc) were pooled and concentrated to obtain **46** as a colorless oil which was used directly in the next step (5.2 g, 51%): ^{1}H NMR ($CDCl_3$) δ 0.9 (t, 3H, CH_2CH_3), 2.0 (m, 2H, CH_2CH_3), 3.6 (m, 4H, aliphatic COOMe and CHEt), 3.9 (s, 3H, aromatic COOMe), 7.4−8.0 (m, 4H, aromatic protons).

Step 2. A solution of diester **46** (5.2 g, 22 mmol) in dry DMF (18 mL) was added dropwise to a stirred suspension of NaH (60% w/w in mineral oil, calculated to contain 880 mg, 22 mmol) in dry DMF precooled to −5 °C. The reaction mixture was stirred at 0 °C for 5 min, then cooled to −35 °C, and treated dropwise with a solution of **26**·HBr (2.9 g, 7.3 mmol) in dry DMF (38 mL) while keeping the temperature between −45 and −35 °C during addition. The temperature was allowed to rise to 10 °C and stirring was continued for 2.5 h. Excess NaH was destroyed by addition of dry ice, and the DMF was removed by rotary evaporation (vacuum pump, dry ice/acetone). The residue was partitioned between EtOAc and H_2O, and the organic layer was dried ($MgSO_4$) and evaporated to a yellowish-brown powder (**47**; 2.14 g, 71%) which was used without further purification: ^{1}H NMR (DMSO-d_6) δ 0.9 (t, 3H, CH_2CH_3), 2.0 (m, 2H, $CHCH_2CH_3$), 3.6 (m, 4H, aliphatic COOMe and $CHCH_2$), 3.9 (s, 3H, aromatic COOMe), 6.5 (br s, 2H, NH_2), 8.3 (s, 1H, pteridine 7-H), 7.4−8.0 (m, 4H, aromatic protons).

Step 3. Diester **47** (2 g, 4.9 mmol) was dissolved in DMSO (9 mL) and 5 N NaOH (9.8 mL, 49 mmol) was added. The solution was stirred at room temperature for 5 min, diluted with H_2O (50 mL), then cooled in ice and adjusted to pH 4.0 with 10% AcOH. The precipitate was filtered, washed with H_2O, and dried in vacuo at 60 °C to obtain **49** as a yellow powder (1.12, 60%) which was used without further purification: mp 140 °C, darkening above 120 °C; IR (KBr) ν 3350, 2950, 1710, 1610, 1560, 1540, 1500, 1450, 1390, 1360 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.75 (m, 3H, CH_2CH_3), 1.90 (m, 2H, $CHCH_2CH_3$), 3.61 (s, 3H, aliphatic COOMe), 7.33 (m, 2H, 3′- and 5′-H), 7.77 (m, 2H, 2′- and 6′-H), 8.18 (d, 1H, pteridine 7-H); MS *m*/*z* 397 (M + 1). More vigorous hydrolysis conditions were tried in order to convert **47** to **48** but led to complex mixtures of difficultly separable products.

Step 4. Acid **49** (280 mg, 0.70 mmol) was dissolved in dry DMF (15 mL) with gentle warming, and the solution was cooled to 0−5 °C and treated dropwise with *i*-BuOCOCl (226 mg, 1.66 mmol) followed by Et_3N (168 mg, 1.66 mmol). After 1.5 h of stirring, **23**·HCl (510 mg, 1.66 mmol) was added, and stirring was resumed at 0−5 °C for 2 h and continued at room temperature for 24 h. The solvent was removed by rotary evaporation (vacuum pump, receiver cooled in dry ice), and the residue was taken up in $CHCl_3$. The solution was washed with H_2O, dried ($MgSO_4$), and applied onto a silica gel column (20 g, 2 × 20 cm) which was eluted successively with 98:2 and 97:3 $CHCl_3$−MeOH. Fractions giving a single TLC spot with R_f 0.4 (silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated, and the residue was taken up in a minimum of 95:5 $CHCl_3$−MeOH. The solution was added to a large excess of Et_2O, and the precipitated solid was filtered, washed with Et_2O, and dried in vacuo at 60 °C to obtain **50** as a yellow solid (87 mg, 20%): mp 140 °C, darkening above 120 °C; IR (KBr) ν 3350, 2950, 1710, 1610, 1560, 1540, 1500, 1450, 1390, 1360, 1220 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.75 (m, 3H, CH_2CH_3), 1.60−1.80 (m, 4H, β- and γ-CH_2), 1.95 (m, 2H, CH_2CH_3), 3.56−3.61 (m, 10H, δ-CH_2, 9-CH_2, two COOMe groups), 4.42 (m, 1H, α-CH), 6.5 (br s, 2H, NH_2), 7.33 (m, 2H, 3′- and 5′-H), 7.77 (m, 2H, 2′- and 6′-H), 7.82−7.84 (m, 4H, phthaloyl protons), 8.18 (d, 1H, pteridine 7-H), 8.7 (d, 1H, CONH). Anal. ($C_{33}H_{34}N_8O_7$·$0.8H_2O$) C, H, N.

***N*$^{\alpha}$-[4-[1-(2,4-Diaminopteridin-6-yl)-2-methoxycarbonyl-2-butyl]benzoyl]-*N*$^{\delta}$-hemiphthaloyl-L-ornithine (18).** Diester **50** (50 mg, 0.076 mmol) was dissolved in DMSO (0.5 mL), 1 N NaOH (0.26 mL, 0.26 mmol) was added, and the reaction mixture was stirred at room temperature for exactly 5 min, then diluted with H_2O (8 mL) and adjusted to pH 4.0 with 10% AcOH. The precipitate was filtered, washed with H_2O, and dried in vacuo at 60 °C to obtain **18** as a pale-yellow powder (31 mg, 61%): mp >190 °C dec, darkening above 170 °C; HPLC 30 min (C_{18} silica gel, 12% MeCN in 0.1 M NH_4-OAc, pH 6.8, 1 mL/min); IR (KBr) ν 3420, 2950, 1715, 1640, 1550, 1500, 1450, 1360, 1300, 1220 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.75 (m, 3H, CH_2CH_3), 1.58 (m, 2H, β-CH_2), 1.89−1.95 (m, 4H, CH_2CH_3 and γ-CH_2), 3.2 (m, 2H, δ-CH_2), 3.55 (m, 2H, 9-CH_2), 3.59 (s, 3H, aliphatic COOMe), 4.38 (m, 1H, α-CH), 6.5 (br s, 2H, NH_2), 7.33 (m, 2H, 3′- and 5′-H), 7.33−7.78 (m, 4H, phthaloyl ring protons), 7.84 (m, 2H, 2′- and 6′-H), 8.15 (d, 1H, pteridine 7-H), 8.7 (d, 1H, CONH); MS *m*/*z* 659 (M + 1). Anal. ($C_{32}H_{32}N_8O_8$·$1.5H_2O$) C, H, N.

4-(Methoxycarbonyl)propiophenone (51) Step 1. A mixture of *p*-bromopropiophenone (**52**; 5 g, 23 mmol) and CuCN (2.4 g, 27 mmol) in dry DMF (6 mL) was refluxed for 4 h, then poured into H_2O (15 mL) containing $FeCl_3$ (9.2 g) and 12 N HCl (2.3 mL). The mixture was kept in a water bath at 70 °C for 20 min and left at room temperature overnight. The precipitated solid was filtered and dissolved in toluene, and the solution was washed with 1 N HCl, H_2O, and 10% NaOH. The organic layer was dried ($MgSO_4$) and evaporated to obtain 4-propionylbenzonitrile (**53**; 2.9 g, 79%).

Step 2. The foregoing nitrile (5 g, 31 mmol) was dissolved in 2-methoxyethanol (100 mL), 2 N NaOH (100 mL) was added, the solution was heated in an oil bath at 100 °C for 15 h, and the reaction mixture was cooled in ice and adjusted to pH 7−8 with 12 N HCl. The precipitate was filtered, washed with H_2O, and redissolved in dilute ammonia at pH 8−9. A small amount of undissolved material was filtered off, and the filtrate was adjusted to pH 2 with 12 N HCl. The solid was collected, washed, and dried in vacuo to obtain 4-carboxypropiophenone (**54**; 4.4 g, 78%): mp 172−178 °C dec, softening above 165 °C (lit.[23] mp 157−158 °C, lit.[40] mp 176−178 °C).

Step 3. A solution of **54** (4.4 g, 25 mmol) in dry DMF (30 mL) was treated with *i*-Pr_2NEt (4.1 g, 32 mmol) and MeI (4.9 g, 35 mmol), and the mixture was stirred at room temperature for 40 h. The solvent was removed by rotary evaporation (vacuum pump, dry ice/acetone), and the residue was stirred with 3% Na_2CO_3 (40 mL), filtered, washed with H_2O, dried in vacuo, and recrystallized from MeOH to obtain **51** as a colorless solid (4.2 g, 89%): mp 80−81 °C (lit.[23] mp 80−81 °C). This product was used directly in the next reaction.

Methyl 2-L-[4-[1-(2,4-Diaminopteridin-6-yl)-2-butyl]-benzoyl]amino-5-phthalimidopentanoate (58). Step 1. *n*-Bu_3P (1.82 g, 3 mmol) was added to a solution of **26**·HBr (1 g, 3 mmol) in dry DMSO (35 mL) at 55 °C, and the mixture was stirred under N_2 for 30 min and then cooled back to room temperature. Keto ester **51** (573 mg, 3 mmol) was added, followed by NaH (60% in mineral oil, calculated to contain 240 mg, 6 mmol). The mixture turned red as H_2 was evolved. Stirring at room temperature was continued for 40 h, and the solvent was removed by rotary evaporation (vacuum pump, dry ice/acetone). The residue was stirred in Et_2O, and the orange solid was filtered, washed with H_2O, and dried to obtain **55** as a yellow solid (650 mg, 62%) which was used directly in the next step: mp >235 °C, darkening above 200 °C; MS *m*/*e* 351 (M + 1); IR (KBr) ν 3450, 3350, 3200, 2950, 1720, 1615, 1565, 1555, 1440, 1350, 1285 cm^{-1}; ^{1}H NMR (DMSO-d_6) cis isomer δ 1.04 (t, CH_2CH_3), 2.57 (q, CH_2CH_3), 3.84 (s, COOMe), 6.64 (s, vinyl H), 7.34 (d, 3′ and 5′-H), 7.75 (d, 2′- and 6′-H), 8.06 (s, pteridine 7-H), trans isomer δ 1.10 (t, CH_2CH_3), 3.16 (q, CH_2CH_3), 3.86 (s, COOMe), 6.98 (s, vinyl H), 7.95 (d, 3′- and 5′-H), 7.98 (d, 2′- and 6′-H), 8.79 (s, pteridine 7-H).

Step 2. PtO_2 (0.16 g) was added to a solution of **55** (600 mg, 1.7 mmol) in glacial AcOH (85 mL) and the mixture was hydrogenated in a Parr apparatus at (initial pressure 45 lb/in.^{2}) for 18 h. The reaction mixture was filtered through Celite and the filtrate treated with 3% H_2O_2 (2.1 mL) and stirred for 5 h. A second portion of 3% H_2O_2 (1 mL) was added, and stirring was resumed for another 15 h. The solution was concentrated to a volume of 10 mL under reduced pressure, and H_2O (60 mL) was added, followed by dropwise addition

28% NH_4OH to pH 7.5. The precipitate was filtered, washed with H_2O, and dried in vacuo to obtain **56** as a brown solid (510 mg, 85%) which was used directly in the next step: mp >215 °C dec; MS *m/e* 353 (M + 1); IR (KBr) ν 3450, 3350, 2950, 1720, 1620, 1585, 1560, 1440, 1280 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.73 (t, 3H, CH_2CH_3), 1.66 (m, 2H, CH_2CH_3), 3.05−3.18 (m, 3H, 9-CH_2 and CHEt), 3.79 (s, 3H, COOMe), 6.48 (br s, 2H, NH_2), 7.33 (m, 2H, 3′- and 5′-H), 7.80 (m, 2′- and 6′-H), 8.32 (s, 1H, pteridine 7-H). The CONH proton and other NH_2 protons were not observed.

Step 3. A solution of **56** (500 mg, 1.42 mmol) in DMSO (15 mL) was treated with 5 N NaOH (2.8 mL) and stirred at room temperature for 10 min. The reaction mixture was diluted to 100 mL with H_2O, adjusted to pH 4.0 with 10% AcOH, and refrigerated overnight. The precipitate was filtered, washed with H_2O, and dried to obtain **57** as a brown solid (325 mg, 68%) which was used directly in the next step: mp >200 °C dec; MS *m/e* 339 (M + 1); IR (KBr) ν 3420, 3200, 2980, 2910, 1620, 1555, 1440, 1370, 1260 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.72 (t, 3H, CH_2CH_3), 1.66 (m, 2H, CH_2CH_3), 3.08 (m, 3H, 9-CH_2 and CHEt), 6.49 (br s, 2H, NH_2), 7.31 (m, 2H, 3′- and 5′-H), 7.79 (m, 2H, 2′- and 6′-H), 8.32 (s, 1H, pteridine 7-H).

Step 4. Et_3N (363 mg, 3.6 mmol) and *i*-BuOCOCl (125 mg, 0.9 mmol) were added to a stirred solution of **57** (300 mg, 0.89 mmol) in dry DMF (20 mL), followed 30 min later by **23**·HCl (509 mg, 1.57 mmol). After 18 h the solvent was removed by rotary evaporation (vacuum pump, dry ice/acetone), and the residue was taken up in $CHCl_3$. The solution was washed with H_2O, and the organic layer was dried ($MgSO_4$) and evaporated to dryness. The residue was redissolved in 9:1 $CHCl_3$−MeOH and chromatographed on silica gel (flash grade, 20 g, 2 × 20 cm) with 98:2 and 97:3 $CHCl_3$−MeOH as the eluents. TLC homogeneous fractions (R_f 0.38, silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated, and the residue was redissolved in a minimum volume of 9:1 $CHCl_3$−MeOH. The solution was poured into excess Et_2O, and the precipitate was filtered, washed with Et_2O, and dried in vacuo at 60 °C to obtain **58** as a yellow solid (152 mg, 29%): mp 155 °C, softening above 120 °C; IR (KBr) ν 3400, 2950, 1710, 1620, 1550, 1535, 1500, 1440, 1355, 1210 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.72 (t, 3H, CH_2CH_3), 1.61−1.78 (m, 6H, β- and γ-CH_2, CH_2CH_3), 3.06−3.17 (m, 3H, 9-CH_2 and CHEt), 3.59 (m, 5H, δ-CH_2 and COOMe), 4.38 (m, 1H, α-CH), 6.5 (br s, 2H, NH_2), 7.28 (m, 2H, 3′- and 5′-H), 7.5 (br s, 2H, NH_2), 7.68 (m, 2H, 2′- and 6′-H), 7.81−7.85 (m, 4H, phthalimide protons), 8.31 (d, 1H, pteridine 7-H), 8.57 (d, 1H, CONH). Anal. ($C_{31}H_{32}N_8O_5$·H_2O) C, H, N.

***N*$^{\alpha}$-[4-[1-(2,4-Diaminopteridin-6-yl)-2-butyl]benzoyl]-*N*$^{\delta}$-hemiphthaloyl-L-ornithine (17).** A solution of **58** (20 mg, 0.034 mmol) was dissolved in EtOH (10 mL) containing a few drops of H_2O. To the solution were then added $Ba(OH)_2$·$2H_2O$ (21 mg, 0.067 mmol) followed 24 h later by NH_4HCO_3 (53 mg, 0.8 mmol) and the mixture stirred for another 30 min. The reaction mixture was filtered to remove the $BaCO_3$, the EtOH was removed on the rotary evaporator, and the remaining aqueous solution was adjusted to pH 4.0 with 10% AcOH and refrigerated overnight. The solid was isolated by centrifugation, washed with H_2O, and dried in a lyophilizer and a vacuum oven at 60 °C to obtain **17** as a yellow solid (14 mg, 70%): mp >200 °C dec; HPLC 26 min (C_{18} silica gel, 12% MeCN in 0.1 M NH_4OAc, pH 6.8, 1 mL/min); IR (KBr) ν 3400, 2950, 1635, 1530, 1500, 1440, 1370, 1300, 1210 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.7 (t, 3H, CH_2CH_3), 1.55−1.89 (m, 6H, β- and γ-CH_2, CH_2CH_3), 3.06−3.26 (m, 5H, 9-CH_2, CHEt, and δ-CH_2), 4.35 (m, 1H, α-CH), 6.5 (br s, 2H, NH_2), 7.29 (m, 2H, 3′- and 5′-H), 7.38−7.52 (m, 3H, phthaloyl 3-, 4-, and 5-protons), 7.63−7.75 (m, 3H, 3′- and 5′-H, phthaloyl 6-H), 8.27 (m, 1H, phthaloyl CONH), 8.31 (d, 1H, pteridine 7-H), 8.47 (d, 1H, benzoyl CONH). Anal. ($C_{30}H_{32}N_8O_6$·$0.1CH_3COOH$·$3.2H_2O$) C, H, N.

Methyl 2-L-(4-Nitro-1-naphthoyl)amino-5-phthalimidopentanoate (60). 4-Nitro-1,8-naphthoic anhydride (10 g, 0.041 mol) and a solution of HgO (10 g, 0.046 mol) in a mixture of H_2O (25 mL) and AcOH (8 mL) were added with stirring to a solution of NaOH (5.4 g, 0.67 mol) in H_2O (200 mL). The reaction mixture was refluxed for 50 h and cooled. The precipitate was filtered, washed with H_2O, and suspended in 12 N HCl (85 mL). The mixture was refluxed for 3 h, cooled, and filtered. The solid was washed with H_2O and recrystallized from AcOH to obtain brown crystals of 4-nitro-1-naphthoic acid (5.9 g, 66%): mp 218−219 °C (lit.[41] mp 225−226 °C). The crude acid was combined with $SOCl_2$ (25 mL) and 2 drops of dry DMF, and the reaction mixture was refluxed for 20 h. Excess $SOCl_2$ was removed by rotary evaporation and the residue was recrystallized from 1:1 hexanes−Et_2O to obtain the crude acid chloride **59** (4.5 g, 69%). Without further purification the acid chloride (3 g, 0.013 mol) and **23**·HCl (4 g, 0.013 mol) were combined in CH_2Cl_2 (100 mL). The mixture cooled in an ice bath while Et_3N (2.63 g, 0.026 mol) was added dropwise with stirring. The solids dissolved, and stirring was continued for 1 h at 0 °C and for 20 h at room temperature. The reaction mixture was washed successively with 0.1 N HCl, H_2O, and saturated aqueous $NaHCO_3$, then dried ($MgSO_4$) and evaporated. Recrystallization from absolute EtOH afforded **60** as pale-yellow crystals (4.41 g, 71%; 32% overall from 4-nitro-1,8-naphthoic anhydride): mp 144−145 °C; IR (KBr) ν 3250, 1740, 1705, 1650, 1520, 1400, 1330, 1250, 1200 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.75−1.80 (m, 4H, β- and γ-CH_2), 3.61−3.63 (m, 2H, δ-CH_2), 3.71 (s, 3H, OMe), 4.5 (m, 1H, α-CH), 7.6 (d, 1H, naphthoyl 5-H), 7.69−7.85 (m, 6H, naphthoyl 2- and 8-H, 4 phthalimide ring protons), 8.24−8.32 (m, 3H, naphthoyl 6- and 7-H, CONH), 9.1 (d, 1H, naphthoyl 3-H). Anal. ($C_{25}H_{21}N_3O_7$) C, H, N.

Methyl 2-L-(4-Amino-1-naphthoyl)amino-5-phthalimidopentanoate (61). Ammonium formate (2.44 g, 0.039 mmol) and 10% Pd−C (500 mg) were added to a suspension of **60** (4 g, 8.4 mmol) in MeOH. After being stirred at room temperature for 3.5 h, the reaction mixture was filtered through a bed of Celite, the filtrate was evaporated to dryness, and the residue was stirred in cold H_2O, filtered, and dried in vacuo at 60 °C to obtain **61** (0.95 g, 25%): mp 157−160 °C after recrystallization from EtOAc−cyclohexane; IR (KBr) ν 3435, 2950, 1730, 1710, 1645, 1575, 1515, 1400, 1210 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.74−1.83 (m, 4H, β- and γ-CH_2), 3.32−3.68 (m, 5H, δ-CH_2 and OMe), 4.4 (m, 1H, α-CH), 6.1 (br s, 2H, NH_2), 6.6 (d, 1H, naphthoyl 3-H), 7.37−7.44 (m, 3H, naphthoyl 2-, 6-, and 7-H), 7.82−7.83 (m, 4H, phthaloyl ring protons), 8.08 (m, 1H, CONH), 8.38 (m, 1H, naphthoyl 5-H), 8.43 (m, 1H, naphthoyl 8-H). Anal. ($C_{25}H_{23}N_3O_5$·$0.2H_2O$) C, H, N.

Methyl 2-L-[4-[*N*-[(2,4-Diaminopteridin-6-yl)methyl]-amino]-1-naphthoyl]amino-5-phthalimidopentanoate (62). A solution of **61** (445 mg, 1 mmol) and **26**·HBr (360 mg, 0.9 mmol) in dry DMF (5 mL) was stirred at room temperature under N_2 for 5 days in a flask wrapped in aluminum foil. The reaction mixture was then added slowly with constant stirring to a solution of Na_2CO_3 (110 mg) in H_2O (50 mL). The yellow precipitate was filtered, washed with H_2O, dried in a vacuum oven, and chromatographed on a silica gel column (30 g, 2 × 28 cm) that was packed and eluted with 91:9 $CHCl_3$−MeOH. Fractions giving a single TLC spot (R_f 0.36, silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated, the residue was redissolved in a minimal volume of 9:1 $CHCl_3$−MeOH, and the solution was poured into a large volume of Et_2O. The precipitate was filtered, washed with Et_2O, and dried in vacuo to obtain **62** as a yellow solid (0.28 g, 51%): mp 165 °C, darkening above 150 °C; IR (KBr) ν 3430, 2950, 1710, 1610, 1580, 1520, 1440, 1390, 1350, 1250 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.71−1.76 (m, 4H, β- and γ-CH_2), 3.59 (m, 2H, δ-CH_2), 3.63 (s, 3H, OMe), 4.45 (m, 1H, α-CH), 4.67 (m, 2H, 9-CH_2), 6.52 (d, 1H, naphthoyl 3-H), 6.62 (br s, 2H, NH_2), 7.41 (d, 1H, 10-NH), 7.47−7.49 (m, 2H, naphthoyl 6- and 7-H), 7.71 (br s, 2H, NH_2), 7.80−7.83 (m, 4H, phthaloyl ring protons), 8.29−8.32 (m, 2H, naphthoyl 5- and 8-H), 8.46 (d, 1H, CONH), 8.69 (s, 1H, pteridine 7-H). Anal. ($C_{27}H_{29}N_9O_5$) C, H, N.

***N*$^{\alpha}$-2-L-[4-[*N*-[(2,4-Diaminopteridin-6-yl)methyl]amino]-1-naphthoyl]-*N*$^{\delta}$-hemiphthaloyl-L-ornithine (19).** Ester **62** (50 mg, 0.081 mmol) was added to EtOH (25 mL) containing a few drops of H_2O, the mixture was solubilized with the help of a sonication bath, and a solution of $Ba(OH)_2$ (51 mg, 0.16

mmol) in H_2O (25 mL) was added. Stirring was continued for 24 h, solid NH_4HCO_3 (13 mg, 0.16 mmol) was added, and stirring was continued for another 30 min. The $BaCO_3$ precipitate was filtered and the EtOH removed by rotary evaporation. The pH was adjusted to 4.0 with 10% AcOH, the mixture was kept in the refrigerator overnight, and the solid was filtered, washed with H_2O, and dried to obtain **19** as a yellow solid (36 mg, 71%): mp >200 °C dec, darkening above 180 °C; HPLC 15 min (C_{18} silica gel, 10% MeCN in 0.1 M NH_4-OAc, pH 6.8, 1 mL/min; IR (KBr) ν 3420, 2950, 1640, 1570, 1520, 1440, 1370 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.61−1.63 (m, 4H, β- and γ-CH_2), 3.19 (m, 2H, δ-CH_2), 4.36 (m, 1H, α-CH), 4.66 (m, 2H, 9-CH_2), 6.52 (d, 1H, naphthoyl 3-H), 6.53 (br s, 2H, NH_2), 7.34 (m, 1H, naphthoyl 2-H), 7.37 (d, 1H, 10-NH), 7.34−7.51 (m, 5H, naphthoyl 6-H and 7-H, and 3 phthaloyl ring protons), 7.69 (br s, 2H, NH_2), 7.71 (m, 1H, 1 phthaloyl ring proton), 8.25−8.39 (m, 4H, naphthoyl 5′- and 8′-H, naphthoyl and phthaloyl CONH), 8.69 (s, 1H, pteridine 7-H). Anal. ($C_{31}H_{29}N_9O_6 \cdot 0.4CH_3COOH \cdot H_2O$) C, H, N.

Methyl *S*-(Phthalimidomethyl)cysteinate Tosylate Salt (67·TsOH). Step 1. A suspension of L-cysteine methyl ester hydrochloride (**63**·HCl; 1.08 g, 6.30 mmol) in EtOAc (30 mL) was treated with di-*tert*-butyl dicarbonate (1.37 g, 6.30 mmol) followed by Et_3N (0.657 g, 904 μL, 6.50 mmol). After being refluxed for 20 h, the reaction mixture was cooled and extracted with 3% citric acid, H_2O, 5% $NaHCO_3$, and finally H_2O. Evaporation to dryness and column chromatography on silica gel (flash grade, 20 g, 2 × 15 cm) with 2:1 hexanes−EtOAc as the eluent afforded the Boc derivative **65** as an oil which was used directly in the next step (906 mg, 61%): R_f 0.5 (silica gel, 2:1 hexanes−EtOAc); ^1H NMR ($CDCl_3$) δ 1.43 (s, 9H, t-Bu), 2.97 (m, 2H, CH_2), 3.75 (s, 3H, OMe), 4.54 (m, 1H, α-CH).

Step 2. A solution of the foregoing oil (306 mg, 1.30 mmol) in MeOH (10 mL) was treated with *N*-(bromomethyl)phthalimide (313 mg, 1.30 mmol) and *i*-Pr_2NEt (268 mg, 226 μL, 1.30 mmol), and the mixture was refluxed for 30 min, cooled, and washed with 3% citric acid. Evaporation of the solvent and column chromatography on silica gel (flash grade, 20 g, 2 × 15 cm) with 2:1 hexanes−EtOAc as the eluent afforded **66** as a gum (448 mg, 87%) which was used directly in the next step: ^1H NMR ($CDCl_3$) δ 1.42 (s, 9H, t-Bu), 3.15 (m, 2H, β-CH_2), 3.74 (s, 3H, OMe), 4.65 (m, 1H, α-CH), 4.78 (s, 2H, SCH_2N), 7.83 (m, 4H, aromatic protons). When the reaction was carried out in EtOH at 65 °C for 15 min the yield was only 37%, suggesting a marked solvent effect on the rate.

Step 3. A solution of **66** (520 mg, 1.32 mmol) in toluene (25 mL) was treated with *p*-TsOH·H_2O (257 mg, 1.35 mmol), refluxed for 10 min, and cooled to room temperature until a precipitate formed. The solid was filtered, washed with toluene, and dried in vacuo at 65 °C over P_2O_5 to obtain **67**·TsOH as a white solid (527 mg, 86%; 46% overall from **63**·HCl): mp 160−162 °C; IR (KBr) ν 3460, 1755, 1710, 1610, 1520, 1510, 1465, 1440, 1415, 1385, 1310, 1285, 1215 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.30 (s, 3H, Me of TsOH), 3.21 (m, 2H, β-CH_2), 3.72 (s, 4H, OMe, partially overlapped by H_2O signal), 4.35 (m, 1H, α-CH), 4.78 (s, 2H, SCH_2N), 7.13 (d, J = 8 Hz, 2H, 3- and 5-H of TsOH), 7.53 (d, J = 8 Hz, 2H, 2- and 6-H of TsOH), 7.93 (s, 4H phthalimide protons), 8.52 (br m, 3H, NH_3^+). Anal. ($C_{20}H_{22}N_2O_7S_2 \cdot 0.5H_2O$) C, H, N, S.

***N*-[4-[(2,4-Diamino-6-pteridin-6-yl)methylamino]benzoyl]-*S*-(2-carboxybenzamidomethyl)-L-cysteine (20). Step 1.** Et_3N (50 mg, 69 μL, 0.5 mmol) and *i*-BuOCOCl (68 mg, 65 μL, 0.5 mmol) were added to a stirred suspension of 4-amino-4-deoxy-N^{10}-formylpteroic acid (**68**; 170 mg, 0.5 mmol) in dry DMF (10 mL) at room temperature. After 5 min, when the solution became clear, **67**·TsOH (233 mg, 0.33 mol) and a second portion of Et_3N (50 mg, 69 μL, 0.5 mmol) were added. Another set of stepwise additions was carried out (23 μL of Et_3N, 22 μL of *i*-BuOCOCl, and 77 mg of **67**·TsOH), and as soon as this addition cycle was completed the solvent was evaporated under reduced pressure and the residue was swirled with Et_2O. The Et_2O was decanted and the insoluble material was partially purified by flash silica gel chromatography (13 g, 2 × 9 cm) with 10:1 $CHCl_3$−MeOH as the eluent. Fractions showing a TLC spot at R_f 0.2 (silica gel, 9:1 $CHCl_3$−MeOH) were combined and diluted with Et_2O until a precipitate formed. The solid was filtered and dried in vacuo at 65 °C over P_2O_5 to obtain the intermediate phthalimide ester **69** (203 mg, >100%). Although the ^1H NMR spectrum contained peaks consistent with the presence of some residual Et_3N·HCl, this solid was used without further purification.

Step 2. A solution of crude **69** (200 mg, assumed to contain the theoretical 0.325 mmol) was dissolved in DMSO (4 mL) and the solution was treated with 2 N NaOH (1.5 mL, 3 mmol). After exactly 2 min of stirring at room temperature, the solution was diluted with H_2O (50 mL) and adjusted to pH 7.5 with saturated aqueous $(NH_4)_2SO_4$. Analytical HPLC (8% MeCN in 0.1 M NH_4OAc, pH 6.9, 1.0 mL/min) showed two peaks with retention times of 15 and 25 min, respectively, and a peak height ratio of ca. 2:1. Treatment of an aliquot of the solution with excess 2 N NaOH at room temperature for 15 min did not alter the ratio. The slower-moving major product was isolated by preparative HPLC using the same eluent system. Homogeneous fractions were pooled and freeze-dried, and the residue was dissolved in dilute ammonia. Acidification with AcOH yielded a precipitate, which was filtered and dried in a lyophilization apparatus to obtain **20** as a yellow solid (24 mg, 7% for two steps): mp >200 °C dec; IR (KBr) ν 3340 br, 1710, 1640, 1600, 1540, 1510, 1445, 1405, 1380, 1325, 1260 cm^{-1}. The identity of the minor product was not investigated. Anal. ($C_{26}H_{25}N_9O_6S \cdot CH_3COOH \cdot 1.5H_2O$) C, H, N, S.

***N*-[4-[(2,4-Diamino-6-pteridin-6-yl)methylamino]benzoyl]-*S*-(2-carboxybenzamidomethyl)-D,L-homocysteine (21).** Using essentially the same procedure as in the preceding experiment, **70**·HCl (172 mg, 0.5 mmol) and **68** (170 mg, 0.5 mmol) were converted into phthalimide ester **72**. After partial purification by flash chromatography on a silica gel (13 g, 2 × 9 cm) with 9:1 $CHCl_3$−MeOH as the eluent, the coupling product was treated with NaOH in DMSO. Preparative HPLC (C_{18} silica gel, 10% MeCN in 0.1 M NH_4OAc, pH 6.9) afforded **21** as a yellow solid (57 mg, 19% for two steps): mp >200 °C dec; HPLC 11 min (C_{18} silica gel, 10% MeCN in 0.1 M NH_4-OAc, pH 6.9, 1.0 mL/min); IR (KBr) ν 3330 br, 3070, 2930, 1635, 1605, 1535, 1505, 1445, 1400, 1325, 1260 cm^{-1}; ^1H NMR (DMSO-d_6 + 1 drop D_2O) δ 2.17 (m, 2H, β-CH_2), 2.73 (m, 2H, γ-CH_2) (partial overlap by DMSO signal), 4.47 (m, 5H, 9-CH_2, α-CH, and SCH_2N), 6.70 (d, J = 8 Hz, 2H, 3′- and 5′-H), 7.63 (m, 6H, 2′-H, 6′-H, and phthaloyl protons), 8.77 (s, 1H, pteridine 7-H). Anal. ($C_{27}H_{27}N_9O_6S \cdot 2H_2O \cdot 0.5CH_3COOH$) C, H, N, S.

Cell Growth Inhibition Assay. CCRF-CEM human leukemic lymphoblasts (American Type Culture Collection, Rockville, MD) were maintained in suspension culture in 175-cm^2 vented flasks at 37 °C in a 5% CO_2 humidified atmosphere in RPMI 1640 medium (Fisher, Boston, MA) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, MO). In the growth inhibition assays, cells (5.5×10^4/mL) were incubated for 72 h in 24-well microtiter plates containing different concentrations of drugs (0.1−1000 nM in half-log increments) or in the absence of drug (negative controls). A 0.5-mL aliquot from each well was suspended in Isoton II (Fisher, Boston, MA) and cell numbers were determined with a hemocytometer after staining with trypan blue or eletronically with a Coulter model Z_{BI} counter gated for a cell diameter of 10−30 μm. In the latter procedure, background values were obtained by counting cells that had been treated for 72 h with 1 μM PT523, a concentration ca. 1000-fold higher than the IC_{50}. Replicate validation trials showed that these cells were completely permeable to trypan blue and that >98% of them had a diameter of <10 μm. The survival fraction (SF) was calculated from the formula: SF = (treated cell number − background)/(control cell number − background). A semilog plot of drug concentration versus SF was generated with Microsoft Excel software, and the IC_{50} was determined by interpolation. Each assay was performed at least three times, and the results were averaged.

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